An investigation of the inhibition of non-typeable *Haemophilus influenzae,* by substances secreted by *Haemophilus haemolyticus*

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Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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The bacterial isolates used in the research investigations that comprise this thesis were from existing culture collections, acquired as part of routine care. As such, their use is exempt from ethical review according to the "National Statement of Ethical Conduct in Human Research" developed by the National Health and Medical Research Council, the Australian Research Council and the Australian Vice-Chancellor's Committee in 2007, because it is both a) negligible risk and b) involves existing collections of non-identifiable data. This exemption status has further been confirmed by the Human Research Ethics Committee of the University of Tasmania.

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Statement of Candidature Contribution to Thesis

This thesis is comprised of research investigations in which the candidate (Roger Latham) was the lead investigator. However, other people contributed significantly. The contribution were as follows:

Roger D Latham (Candidate, School of Medicine, University of Tasmania)

Lead investigator for all research investigations, with responsibility for experimental design, set up and conduct of experiments, data collection, data analysis, and data interpretation. He is the first author on the two manuscripts which were derived directly from the work in this thesis.

Dr Stephen Tristram (Supervisor, School of Health Sciences, University of Tasmania).

General supervision, assistance with experimental design and methods, and revision of thesis.

Dr David Gell (Supervisor, School of Medicine, University of Tasmania)

Assistance with design of experiments, experimental methods, revision of thesis, and conduct and set up of techniques related to protein purification techniques.

Dr Bruce Lyons (Primary supervisor, School of Medicine, University of Tasmania)

General supervision, assistance with experimental design and methods, and revision of thesis.

Contributions to specific chapters

Chapter 4. Co-culture assays, primarily performed by Mr Rory Fairbairn (Student, University of Tasmania), under the supervision of R. Latham.

Chapter 5. Molecular size determination, primarily conducted by D. Gell, with R. Latham assisting.

Chapter 6. SEC and RP-HPLC were primarily conducted by D. Gell, with R. Latham assisting. Cloning and expression were performed by R. Latham under the guidance of D. Gell. Mass spectrometry analysis and database search were entirely conducted by Dr Richard. Wilson (Central Science Laboratory, University of Tasmania).

Chapter 7. Hh-NIS holoprotein and apoprotein fractions were prepared by D. Gell.

We, the undersigned, agree with the above stated “proportion of work undertaken” for each of the chapters which contributed to this thesis.

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Publications and Presentations from Candidature

Publications

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Oral Presentations

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Abstract

Non-typeable *Haemophilus influenzae* (NTHi) are Gram-negative bacteria that reside in the upper respiratory tract of humans. As well as being part of the normal flora, they are opportunistic pathogens, causative of non-invasive respiratory tract infections, and an occasional cause of invasive infections. In the Australian aboriginal population, a high prevalence of otitis media (OM) in children is a factor that contributes significantly to their educational and economic disadvantage, and NTHi is the most important cause of recurrent OM cases. In the worldwide adult population, NTHi causes approximately 25% of the "exacerbations" of chronic obstructive pulmonary disease (COPD), which ultimately lead to death in most cases. NTHi is transmitted by respiratory secretions, initially colonising the pharynx, and in the case of disease, moves to the lower respiratory tract (COPD) or middle ear (OM). Efforts to treat and prevent NTHi infection have been hampered by antibiotic resistance and difficulty in finding suitable vaccine targets, and alternative treatment or prevention strategies are therefore desired. Bacterial therapy (probiotics) for the prevention of respiratory tract infections, based on co-colonizing bacteria, has been a topic of research in the 21st century, and a commercial preparation of *Streptococcus salivarius* (BLIS K12™*)* is now available for the prevention of *S. pyogenes* infections, and another *S. salivarius* preparation (24SMB) is patented for prevention of *S. pneumoniae* infections. However, there have so far been no co-colonizing probiotics developed specifically for the prevention of NTHi infections. *Haemophilus haemolyticus* are bacteria that are closely related to NTHi, also colonize the upper respiratory tract of humans, but are not opportunistic pathogens of the respiratory tract, and only very rarely causative of invasive infections in the immunocompromised host. Recently, pre-treatment of epithelial cells with *H. haemolyticus* was shown to prevent NTHi attachment, and as a result, *H. haemolyticus* was suggested as a possible agent of bacterial therapy (Pickering et al., 2016).

In this project, the NTHi-inhibitory activity of *H. haemolyticus* was investigated, with a focus on secreted substances. Initially, SYBR Green real-time (RTPCR) assays were developed for the identification of the two species, which were then applied to a collection of isolates from Australian pathology laboratories. The *H. haemolyticus* isolates were then screened for inhibition of NTHi, using co-culture and agar well diffusion assays. Two *H. haemolyticus* isolates (BW1 and RHH122) were identified as consistent producers of an NTHi inhibitory substance (NIS), and the physical and chemical properties of the NIS produced by BW1 were characterised and published. The NIS produced by BW1 (BW1-NIS) and RHH122 (RHH122-NIS) were identified, by mass spectrometry analysis and comparison with published whole genome sequences, as identical to a putative 27 kDa OMPA-like protein in *H. haemolyticus* isolate M19107. A recombinant version of this *H. haemolyticus* NIS (Hh-NIS), made in *E. coli* from the *Hh-NIS* ORF without signal peptide, also inhibited NTHi. With *in silico* structural modelling, similarities were identified between Hh-NIS and the haemoglobin-haptoglobin utilisation protein A (hpuA) in *Kingella denitrificans* and *Neisseria gonohorreae*. The spectrum of absorbance of Hh-NIS identified haem as a likely component, and with cation exchange chromatography, Hh-NIS was separated into a holoprotein fraction, containing haem as a ligand, and an apoprotein fraction, from which haem was absent. As the activity of both fractions against NTHi was similar, and the addition of haemin neutralised the activity of Hh-NIS, haem was considered not to be a co-factor required for activation of an enzyme, but that it had another role. The addition of purified Hh-NIS holoprotein to broth cultures, resulted in inhibition of NTHi growth, and promotion of growth of an Hh-NIS producing strain of *H. haemolyticus* (BW1), but only in haemin-limited conditions. A model mechanism for activity was described, whereby Hh-NIS is a haemophore that binds haem, making it available to producing strains, but unavailable to NTHi. To further understand the biological function and importance of Hh-NIS to *H. haemolyticus,* construction and testing of a knockout mutant, the

elucidation of the control of Hh-NIS secretion and utilisation, and *in vivo* testing in animal models are recommended. With regards to its application as a probiotic for NTHi control in humans, in the continued absence of an effective vaccine, *in vivo* testing of *H. haemolyticus* strains which produce Hh-NIS is recommended, although as haem-acquisition genes are associated with pathogenicity, a cautionary approach should be taken.

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List of Non-standard Abbreviations

0.5xsBHIA	Half-strength supplemented brain heart infusion agar
BLNAR	β-lactamase negative ampicillin resistant
BW1	A NIS producing isolate of *H. haemolyticus*
BW1-NIS	The NIS produced by BW1
CA	Chocolate agar
COPD	Chronic obstructive pulmonary disease
DPBS	Dulbecco's phosphate buffered saline
Hh-hpd	hpd in *H. haemolyticus*
Hh-NIS	The gene for Hh-NIS
Hh-NIS	The NIS produced by *H. haemolyticus*, as identified in this thesis.
Hib	*H. influenzae* type b
Hi-hpd	hpd in *H. influenzae*
HRM-PCR	High-resolution melt PCR
MIC	Minimum inhibitory concentration
NAD	Nicotinamide adenine dinucleotide
NCTC 11315	A strain of NTHi, used throughout the thesis
NCTC 4560	A strain of NTHi, used throughout the thesis
NIS	NTHi inhibiting substance
NTHi	Non-typeable *Haemophilus influenzae*
OM	Otitis media
ORF	Open reading frame
PCV	*Streptococcus pneumoniae* polysaccharide conjugate vaccine
PHiD-CV	Pneumococcal conjugate vaccine, with *H. influenzae* protein D as carrier
RHH122	A NIS producing isolate of *H. haemolyticus*
RHH122-NIS	The NIS produced by RHH122
RP-HPLC	Reverse-phase high-performance liquid chromatography
RTPCR	Real time polymerase chain reaction
sBHIB	Supplemented brain heart infusion broth
SEC	Size exclusion chromatography
SMM	Skim milk media
sTSB	Supplemented tryptone soya broth

Chapter 1.

Literature Review

1.1 *Haemophilus influenzae*

Haemophilus influenzae are Gram-negative, facultatively anaerobic, coccobacillary bacteria, with the upper respiratory tract of humans their reservoir. These bacteria are nutritionally fastidious, and require nicotinamide adenine dinucleotide (NAD, V factor), along with a source of haem (X factor), which may also be provided in the form of haemin, haematin or haemoglobin. *H. influenzae* can be divided into typeable and non-typeable forms depending on the presence or absence of a polysaccharide capsule, with the capsulated (typeable) forms classified into one of six serotypes (a to f) based on the reactivity of antisera with their capsule (Pittman, 1933).

Although *H. influenzae* are frequent colonizers of healthy children and adults (Lemon et al., 2010; Murphy et al., 2009), they are also an important cause of disease. The most clinically relevant serotype is *H. influenzae* type b (Hib), which causes serious invasive infections, including meningitis and pneumonia, particularly in children and infants. With the introduction in the 1980s of routine immunisation with a polyribosylribitol phosphate (type b capsular polysaccharide) vaccine, and then a conjugate version also in the 1980s and beyond, the incidence of invasive infection due to this serotype has dramatically decreased, such that the risk in developed countries is now almost negligible (Peltola, 2000). Still, the risk of Hib infection remains high in non-vaccinated populations, particularly in developing countries (Obonyo and Lau, 2006). However, the focus of *H. influenzae* research has now shifted to the non-typeable *H. influenzae* (NTHi).

1.2 Non-typeable *Haemophilus influenzae* (NTHi)

After the introduction of routine vaccination against Hib, and the polysaccharide conjugate vaccine (PCV) against *Streptococcus pneumoniae* in the early 2000s, the global impact on health due to Hib and *S. pneumoniae* infections was reducing, but the relative and absolute

impact due to NTHi infections was increasing. The main explanation given for the change, was the "strain replacement" of Hib and *S. pneumoniae* by NTHi (Biesbroek et al., 2014; Langereis and de Jonge, 2015; Spijkerman et al., 2012). NTHi and non-vaccine-preventable *S. pneumoniae* are now the leading causes of otitis media in children (Ngo et al., 2016), and exacerbations of chronic obstructive pulmonary disease (Rosell et al., 2005; Sethi and Murphy, 2008). It is now also the case that NTHi have become increasingly important in bacterial conjunctivitis, bacterial sinusitis, exacerbations of cystic fibrosis, persistent bacterial bronchitis, and pneumonia (Van Eldere et al., 2014). Additionally, NTHi have replaced Hib as the leading cause of invasive infections for this species, and there is a particularly high incidence in infants, the indigenous and the elderly (Adam et al., 2010; Resman et al., 2011; Van Eldere et al., 2014; Wan Sai Cheong et al., 2015; Whittaker et al., 2017).

1.3 Antimicrobial therapy

Despite some NTHi infections such as simple otitis media being self-limiting, most NTHi infections are managed with antimicrobial therapy. The most commonly used agents are β-lactams, including aminopenicillins alone or in combination with a β-lactamase inhibitor, and cephalosporins (Tristram et al., 2007). Although most macrolides have relatively low activity against NTHi (Jacobs et al., 2003), the newer and more active derivatives, such as azithromycin, have occasional application due to their broad spectrum activity against respiratory pathogens, as well as their immunomodulatory and anti-inflammatory effects (Kovaleva et al., 2011; Sriram et al., 2017) Although most NTHi infections can be managed with antimicrobials, resistance is an increasing problem.

1.4 Antimicrobial resistance

Resistance to β-lactams is widespread in NTHi, and may be chromosomally or plasmid encoded. In NTHi, high-level resistance to aminopenicillins is conferred by plasmid encoded

TEM-1 and ROB-1 β-lactamases. Although the prevalence of this type of resistance is quite variable, in most regions it is 20-30% (Farrell et al., 2005; Gilsdorf, 2015; Tristram et al., 2007). Of concern is the increasing prevalence of NTHi strains with a chromosomally encoded altered penicillin binding protein 3 (Dabernat and Delmas, 2012). These strains, referred to as β-lactamase negative ampicillin resistant (BLNAR), have decreased susceptibility to all β-lactams, and their prevalence in most parts of the world is around 20%, but is more than 50% in some areas including Japan (Wajima et al., 2016). Although this resistance was initially considered to have arisen only from *de novo* mutations, it is has been shown to be transferable between strains via transformation and recombination, (Søndergaard et al., 2015; Takahata et al., 2007; Witherden et al., 2014) which may account for the rapid rise in prevalence.

Resistance to macrolides is common in NTHi, yet they are still in use. Although most NTHi have an AcRAB type efflux pump (Sanchez et al., 1997), which results in low-level resistance to macrolides (Pettigrew et al., 2016), they are still usually susceptible at clinically relevant concentrations (Cameron et al., 2012; Sriram et al., 2017; Tristram et al., 2007). The existence of high-level resistance, conveyed by transferable acquired macrolide resistance genes of the erm and mef types, has been demonstrated in NTHi (Roberts et al., 2010), but its importance is disputed (Atkinson et al., 2015; Pettigrew et al., 2016; Seyama et al., 2016). However, the value of macrolide therapy would likely be greatly diminished if these genes do become established in NTHi, as they have done in Gram-positive bacteria.

The reliability of antimicrobial therapy for treatment of NTHi infections is clearly being diminished by an increase in the spectrum and prevalence of resistance, and combined with its increasing importance as a pathogen, has resulted in NTHi being entered onto a “priority

pathogens list" by the World Health Organisations, for guidance into research, discovery and development of new antibiotics (World Health Organisation, 2017).

1.5 Vaccines

Despite being a topic of research for more than three decades, there is still no effective vaccine for protection against NTHi infection. Inactivated whole-cell NTHi oral vaccines have been tested extensively (Sriram et al., 2017), but their efficacy has been only moderate or insignificant (Clancy et al., 2016; Teo et al., 2017).

More recent efforts have focused on the use of specific antigens, and a list of those under study has been compiled (Khan et al., 2016; Murphy, 2015). Confounding factors in the use of these antigens for vaccine development have been the absence of a capsular target, phase variability (Atack et al., 2015; Fox et al., 2014; VanWagoner et al., 2016) and the extreme heterogeneity of NTHi isolates (Eutsey et al., 2013), and as yet, no single antigen has been discovered that is characteristic of all pathogenic strains (Cerquetti and Giufrè, 2016). However, protein D (hpd) has been incorporated as a carrier into the currently available pneumococcal conjugate vaccine (PHiD-CV), and some efficacy against NTHi carriage and disease rates has been demonstrated. In a randomised double-blind pneumococcal otitis efficacy trial (POET), 11Pn-PD, the prototype vaccine for PHiD-CV, reduced the number of cases of acute OM caused by NTHi (Prymula et al., 2006), as well as the carriage rate (Prymula et al., 2009). Then, in Australian aboriginal communities, PHiD-CV in place of 7-valent PCV was associated with a reduction in suppurative OM (Leach et al., 2014), which was then linked to a reduction in NTHi in the ear discharge (Leach et al., 2015). Later, in the same communities, with the transition from PHiD-CV to a 13-valent PCV (without hpd), the presence of NTHi in the OM ear discharge was increased (Leach et al., 2016). However, a concern with the use of hpd as the only NTHi antigen in a vaccine , is the existence of a subset of disease-associated true NTHi strains that

are clearly deficient in hpd (Smith-Vaughan et al., 2014), and while none of the strains were from PHiD-CV vaccinated individuals, a concern remains that continued use of the vaccines would positively select for these organisms. Were such an increase in the prevalence of hpd-deficient NTHi to occur, any efficacy of the vaccines against NTHi would likely be reduced. However these concerns may be an overstatement, as a different study has shown that hpd-deficient NTHi mutants were approximately 100-fold less virulent (Janson et al., 1994).

Although researchers are hopeful that a vaccine with substantial efficacy will be developed in the next decade (Khan et al., 2016; Murphy, 2015), there is no certainty that it will happen, and the development of a non-antibiotic, non-vaccine approach to controlling NTHi infection should be considered.

1.6 Microbial interference

The deliberate introduction of commensal bacteria that are antagonistic to pathogens, is an approach that has had extensive research, as well as discussion in the medical and wider communities. This “microbial interference” approach, or “probiotics” as it is more commonly known, is normally associated with the ingestion of live lactic acid bacteria, for their beneficial effects, perceived or otherwise, in the gastrointestinal tract, or the immune system in general. However, in parallel to this research, there exists a substantial although smaller body of research, concerning the use of microbial interference for the prevention or treatment of infections in the upper respiratory tract. Reviews of this topic (Brook, 2005; Esposito et al., 2014; Hao et al., 2015; Marom et al., 2016; Popova et al., 2012; Vouloumanou et al., 2009) show the undoubtable focus of this research has been on the the prevention or treatment of *S. pyogenes* and *S. pneumoniae* infections, or the non-specific immune-modulatory effects of *Lactobacillus* spp. and *Bifidobacterium* spp.. Although the results are variable, as shown

below, there are a sufficient number of examples of treatment efficacy for this approach to be taken seriously.

A strain of *Streptococcus salivarius* from the throat of a healthy child (Smith et al., 1993), has been developed into the commercially available, orally administered preparation, BLIS K12^{tm}. This strain produces the bacteriocins, salivaricin A2 and salivaricin B (Hyink et al., 2007), with activity against *S. pyogenes.* The effectiveness of BLIS K12^{tm} for prevention of a range of conditions has been reviewed, with results suggestive of efficacy against respiratory tract infections (Zupancic et al., 2017). In all of four small clinical trials (Di Pierro et al., 2012, 2013, 2014; Gregori et al., 2016), BLIS K12^{tm} was protective against pharyngitis and/ or tonsillitis. Also, in small trials of BLIS K12^{tm} for protection against acute otitis media, although less convincing, protective activity was observed (Di Pierro et al., 2015; Power et al., 2008; Walls et al., 2003).

An isolate of *S. oralis* (89a, previously *S. sanguinis* 89a), taken from a healthy child during an outbreak of *S. pyogenes* tonsillitis in Sweden, was shown to have *in vitro* activity against *S. pyogenes* (Grahn and Holm, 1983). Then, in a double-blinded placebo controlled trial on children with chronic secretory OM, 7 of 19 treated with an *S. oralis* 89a nasal spray were cured or much improved, compared with 1 of 17 in the placebo group (Skovbjerg et al., 2009), a significant difference (p=0.044).

A strain of *S. salivarius* (24SMB) with activity against *S. pneumoniae* has been patented for treatment of chronic respiratory tract infections (U.S. Patent No. 20130095044 A1, 2013). When tested *in vitro*, this strain produced a BLIS which inhibited *S. pneumoniae,* (Santagati et al., 2012), and, in a small clinical trial, nasally administered 24SMB colonized the nasopharynx and reduced the rates of paediatric otitis media (Marchisio et al., 2015), although the

differences were insignificant, and conclusions disputed (Di Mario et al., 2016; Marchisio et al., 2016).

Recently, and more convincingly, a retrospective observational study was performed on children with recurrent acute OM, with the treatment consisted of a nasal spray containing *S. salivarius* 24SMB and *S. oralis* 89a (La Mantia et al., 2017). There were 108 participants in the control group and 159 in the treatment group. In the treatment group, there was a significant reduction ($p<0.0001$) in both the number of episodes and severity index of OM.

In an example of a preliminary study, with results that may lead to the development of probiotics for the respiratory tract, *S. aureus* inhibitory activity was examined in a collection of intra-nasal *Staphylococcus* spp. The *S. lugdunensis* isolate IVK28 produced lugdunin, a novel antibiotic with activity against Gram-positive pathogens, including *S. aureus* (Zipperer et al., 2016). To further investigate this, intra-nasal colonisation by *S. aureus* and *S. lugdunensis* was studied in specimens from 187 hospitalised patients. The results showed that carriage of *S. lugdunensis* was associated with a 5.9-fold reduction in *S. aureus* carriage, and that all *S. lugdunensis* isolates were positive for the *lug* operon, and all *S. aureus* were sensitive for lugdunin.

Finally, in one of the few examples of studies on Gram-negative bacteria, intra-nasal inoculation of *Neisseria lactamica* reduced the carriage of *N. meningitidis*. Following on from studies showing carriage and disease of *N. meningitidis* and *N. lactamica* were inversely related (Cartwright et al., 1987; Gold et al., 1978; Olsen et al., 1991), an *N. lactamica* strain (Y92–1009), which had been used for manufacture of an outer membrane vesicle-based vaccine against *N. meningitidis* (Gorringe et al., 2009), was administered intra-nasally to young adults. The individuals treated with the *N. lactamica* experienced an increased rate of

displacement of *N. meningitidis*, as well as a reduced rate of *N. meningitidis* acquisition (Deasy et al., 2015).

As NTHi colonisation of the upper airways is required prior to the development of respiratory tract infections (King, 2012), the inhibition of colonisation is an appealing target for prevention of infection. In the absence of a suitable vaccine, this may be achieved through microbial interference. However, we are unaware of any research on the development of a probiotic directed against NTHi colonisation, but we suggest that *H. haemolyticus* is an agent that should be considered.

1.7 *Haemophilus haemolyticus*

Criteria for a successful probiotic strain include occupation of the same niche as the pathogen, absence of pathogenicity, and inhibition of the pathogen (Dunne et al., 2001). Interestingly, for development of a probiotic against NTHi, Hib strains partially fulfil these criteria in that they occupy the same niche and secrete the bacteriocin "haemocin", which has activity against NTHi (LiPuma et al., 1990, 1992; Venezia and Robertson, 1975). However, Hib would be inappropriate as a probiotic, as they have the potential to cause serious disease in non-vaccinated individuals, and would not establish in the upper respiratory tract of those that have been vaccinated.

H. haemolyticus, like *H. influenzae*, are bacteria that are resident of the upper respiratory tract of healthy adults and children. The two species are genetically and phenotypically closely related, with both dependent on X and V growth factors. However, unlike *H. influenzae*, *H. haemolyticus* is not considered to be an opportunistic pathogen, and although *H. haemolyticus* may be frequently isolated from the respiratory tract of COPD patients, the acquisition of new strains was not associated with exacerbations (Murphy et al., 2007). Nevertheless, *H.*

haemolyticus is a rare cause of invasive disease in the immunocompromised. The species was first described in 1919 (Pritchett and Stillman, 1919), and apart from two cases of endocarditis reported shortly there afterwards (De Santo and White, 1933; Miller and Branch, 1923), the next reported cases of *H. haemolyticus* causing disease were in 2011, with five patients identified with invasive disease, but with no other details given (Jordan et al., 2011). In 2012 there were six reported cases of bacteraemia due to *H. haemolyticus*, along with a single case each of peritonitis and septic arthritis (Anderson et al., 2012; Morton et al., 2012). These 13 cases from 2011 and 2012 were identified from collections of invasive disease causing isolates, which had been initially identified as *H. influenzae*, and their identities had been later re-examined using molecular methods.

Differentiation of *H. haemolyticus* from *H. influenzae* has always been problematic, as it inhabits the same niche, and has much the same phenotypic characteristics. The main characteristic used for differentiating the two organisms has been beta-haemolysis, which is unreliable. In a landmark prospective study of apparent NTHi obtained from various clinical sources in COPD patients, the 12 *H. haemolyticus* isolated from throat swabs were all non-haemolytic, and 50 of 102 isolates of *H. haemolyticus* from sputum were also non-haemolytic (Murphy et al., 2007). To address this problem in *H. haemolyticus* identification, molecular methods have been developed, and are reviewed in the introduction to Chapter 4 of this thesis.

Although *H. haemolyticus* has been described as an emerging pathogen (Jordan et al., 2011), its mechanisms of pathogenicity are poorly understood (Anderson et al., 2012). Typically invasive pathogens (Hib, *S. aureus*, *S. pyogenes*, and *N. meningitidis*) have a capsule, but there are no reports of capsule formation by *H. haemolyticus*, which is also the case for NTHi. The haemolytic characteristic of the organism is the due to the production of a hemolysin,

substances which in *S. aureus* and *S. pyogenes* are virulence factors, but in *H. haemolyticus* the biological role of hemolysin production is unknown (Anderson et al., 2012). Phase-variable phosphoryl choline (ChoP) modification of lipooligosaccharide, is a virulence factor of NTHi for evasion and attachment (Hong et al., 2007; Weiser et al., 1997), encoded by the *lic1* locus. In a study of *lic1* in these two organisms, it was present in 92% of NTHi, and also 43% of *H. haemolyticus* (McCrea et al., 2010a). However, in *H. haemolyticus*, the encoded ChoP was less diverse, which was suggested to be related to reduced virulence. In an *in vitro* investigation of the interaction of host cells with *H. haemolyticus* and NTHi (Pickering et al., 2016), there were clear differences. Although *H. haemolyticus* was less able to attach to and invade nasopharyngeal and bronchoalveolar epithelial cells, exposure to *H. haemolyticus* also resulted in increased cytotoxicity and an inflammatory (IL-6 and IL-8) response, but exposure to NTHi did not. Clearly, despite the similarities of the two organisms, there are differences in how they interact with the host.

The organisms *H. haemolyticus* and *H. influenzae*, both colonize the same locations of the upper respiratory tract, but there is substantial variability between individuals. In a collection of isolates originating from regular monthly morning collection of spontaneously expectorated sputum of COPD patients (Sethi et al., 2002), the NTHi isolates, which had been identified with conventional methods, were re-identified with molecular methods, and 102 of 258 (40%) were *H. haemolyticus*, with the balance *H. influenzae* (Murphy et al., 2007). Also, when the authors re-examined with molecular methods, the 44 NTHi isolates from throat swabs of healthy children in day-care, which had been originally identified by conventional methods which did not differentiate *H. haemolyticus* (Farjo et al., 2004), 12 (27%) were found to be *H. haemolyticus*, and 32 (73%) NTHi. In the same study, Murphy and colleagues provided evidence that *H. haemolyticus* is not a cause of opportunistic respiratory tract infections, when,

in their re-identification of 130 NTHi isolates from middle ear fluids of acute otitis media patients by molecular methods, all were identified as NTHi. Additional retrospective studies of apparent NTHi respiratory isolates have similarly found no evidence that *H. haemolyticus* is a respiratory pathogen, with only 2 out of 480 isolates (Norskov-Lauritsen, 2009), and 7 out of 447 isolates (Zhang et al., 2014), identified as *H. haemolyticus*, and in neither study were the *H. haemolyticus* shown to be of clinical significance.

In a study of *H. influenzae* and *H. haemolyticus* pharyngeal colonisation dynamics, regular throat swabs were taken from four healthy adults over a seven month period, and isolates of both species were identified with molecular methods (Mukundan et al., 2007). Over the whole study, 16% of the isolates were *H. haemolyticus*, and 84% were NTHi, however, between the adults there was extreme variability. From adult B, 236 of 237 (99.6%) isolates were NTHi, and adult D, 48 of 48 (100%) isolates were *H. haemolyticus*, with the results for adults A and C between these two extremes.

As shown above, when *H. haemolyticus* and NTHi are co-inhabitants of the upper respiratory tract, they can do so with the apparent absence of bacterial infection at that particular site. Conceivably, when these bacteria are co-located, due to their common growth requirements, *H. haemolyticus* are controlling the out-growth of NTHi. Such activity would suggest antagonism of NTHi growth by *H. haemolyticus*, and supports a previously described idea, that *H. haemolyticus* may be useful as a bacterial therapy (probiotic) against NTHi infection because it may block colonization (Pickering et al., 2016). The idea had evolved from results in their study, which showed that pre-treatment with *H. haemolyticus* reduced the number of NTHi which could attach to epithelial cells *in vitro*. However the underlying mechanisms for the inhibition of attachment were not investigated.

With the purpose of developing strains of *H. haemolyticus* as probiotics, to supplement antibiotics for the control of NTHi, prior to development of an effective vaccine, we now seek to uncover any inhibitory activity of *H. haemolyticus* toward NTHi, with a focus on secreted substances.

Chapter 2.

General Materials and Methods

2.1 Bacterial collection

The *H. haemolyticus* and NTHi isolates in this study originated from respiratory tract specimens, and were kindly provided by hospital laboratories around Australia. As they were obtained from routine procedures, ethics approval was not required. The isolates were initially identified by colony morphology, Gram-stain, X and V factor dependency, and haemolysis testing, and were subsequently identified by 16S sequencing and RTPCR for species specific markers (Latham et al., 2015).

Twenty Hib isolates, MDU 1 through to MDU 20, were kindly provided by Dr Ben Howden of the Medical Diagnostic Unit of the Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia. Their origins and methods for identification were not disclosed.

The *Haemophilus* reference strains used in our study consisted of eight *H. influenzae* (ATCC 9007, ATCC 43163, ATCC 10211, ATCC 49766, NCTC 8468, NCTC 4560, NCTC 11315, 86-028 NP) and one *H. haemolyticus* strain, ATCC 33390.

Bacterial strains other than *Haemophilus*, as used for sensitivity testing, were kindly provided by Mr David Jones, senior microbiologist, Royal Hobart Hospital, TAS, Australia.

2.2 Culture Media

2.2.1 Chocolate agar (CA)

Chocolate agar (CA) was used for revival, subculturing, and enumeration of *Haemophilus* spp. Plates of CA were prepared by autoclaving a mixture of 19.5g of Columbia blood agar base (Oxoid, CM0331) with 500mL of distilled water at 121°C for 30 minutes, immersion in an 80°C water bath for 30 minutes, mixing in 25mL of defibrinated horse blood (Oxoid, HB250) by swirling for 2 minutes, transferring to a 50°C water bath for 30 minutes, then mixing in half

a vial of resuspended Vitox supplement (Oxoid SR0090A), and pouring 10-15mL in 90mm polystyrene Petri dishes.

2.2.2 Supplemented brain heart infusion broth (sBHIB)

Brain heart infusion (Oxoid, CM1135) was dissolved in distilled water at the rate specified by the manufacturer, autoclaved at 121°C for 30 minutes, cooled to 50°C, then supplemented with HTM supplement (Oxoid, SR0158) and Vitox (Oxoid SR0090A) at the rate recommended by the manufacturer.

2.2.3 Half-strength supplemented brain heart infusion agar (0.5xsBHIA)

For the preparation of half strength supplemented brain heart infusion agar (0.5xsBHIA), half the manufacturer rate of brain heart infusion (Oxoid, CM1135) was mixed with 7.5g/L bacteriological agarose (Oxoid, LP0011), autoclaved at 121°C for 30 minutes, cooled to 50°C, then supplemented with half the manufacturer recommended rates of HTM supplement (Oxoid, SR0158) and Vitox (Oxoid, SR0090A).

2.2.4 Supplemented tryptone soya broth (sTSB)

Cold Filterable Tryptone Soya Broth (Oxoid, CM 1065) was dissolved in distilled water at the manufacturer specified rate, then sterilised by 0.2µm membrane filtration, and supplemented with HTM supplement (Oxoid, SR0158) and Vitox (Oxoid SR0090A) at the rate recommended by the manufacturer, or supplemented with NAD and haemin as specified.

2.2.5 Haemin solution

2 mg/mL haemin (Sigma, 51280) was prepared in 0.1M NaOH by mixing for 220 RPM at 37°C for 1 hour, then sterilization by filtration through a 0.2µm membrane. Aliquots were frozen at -20°C and used on the same day as thawing.

2.2.6 Nicotinamide adenine dinucleotide (NAD) solution

1 mg/mL Nicotinamide adenine dinucleotide (NAD) in water was sterilized by filtering through a 0.2µm membrane. Aliquots were frozen at -20°C and used on the same day as thawing.

2.2.7 Skim milk media (SMM)

Skim milk media (SMM) was used for storage of isolates at -80°C, and consisted of 100g/L skim milk powder (Diploma, Australia) dissolved in Dulbecco's phosphate buffered saline (DPBS, Gibco, 14190-250), which was then autoclaved at 110°C for 15 minutes.

2.3 Incubation of cultures

2.3.1 Solidified media in petri dishes

Incubation was in a humidified atmosphere at 35°C with 5% CO_2 for 16-24 hours.

2.3.2 Agitated Broth

Incubation was in an unmodified atmosphere at 35 to 37°C with 200 RPM agitation. The culture volumes were 20% or less of the manufacturer specified vessel volume, with aluminium foil caps.

2.3.3 Stationary broth

200µL broth volumes were incubated in the wells of lidded 96-well round bottom polystyrene tissue culture clusters (Corning, 3799), in a humidified atmosphere at 35°C with 5% CO_2, for the specified time.

2.4 Storage and revival of cultures

For long term storage, bacterial isolates were grown on CA for 6-12 hours then suspended in SMM to an absorbance of approximately 4 (OD_{600}). Later, cultures were revived from -80°C by incubation on chocolate agar.

2.5 Evaluation of bacterial growth

2.5.1 Viable counts

Ten-fold serial dilutions of bacterial suspensions were prepared in DPBS then 100µL aliquots spread on CA. Colony counts between 30 and 300 were used to calculate the bacterial density in the initial suspension.

2.5.2 Absorbance

Absorbance of bacterial suspensions was in a cuvette with a 10mm path length at a wavelength of 600nm, with a spectrophotometer (Eppendorf, Biophotometer). In the case of experiments with 96 well plates, a microplate reader (Molecular Devices, Spectramax M2) was used, with absorbance measured at a wavelength of 600nm.

2.5.3 Estimation of viable count from absorbance

The biomass of *H. haemolyticus* isolate BW1 in sBHI broth was monitored over a 48 hour period, by measurement of viable counts and absorbance (OD_{600}) (Fig 2.1). For the period between 2.5 hours and 9.3 hours, the growth was logarithmic, and could be represented by the linear equation, log (CFU/mL) = 1.26 (log OD600) + 8.86 (Fig. 2.2), which was used, where indicated, for estimation of viable count from absorbance (OD_{600}).

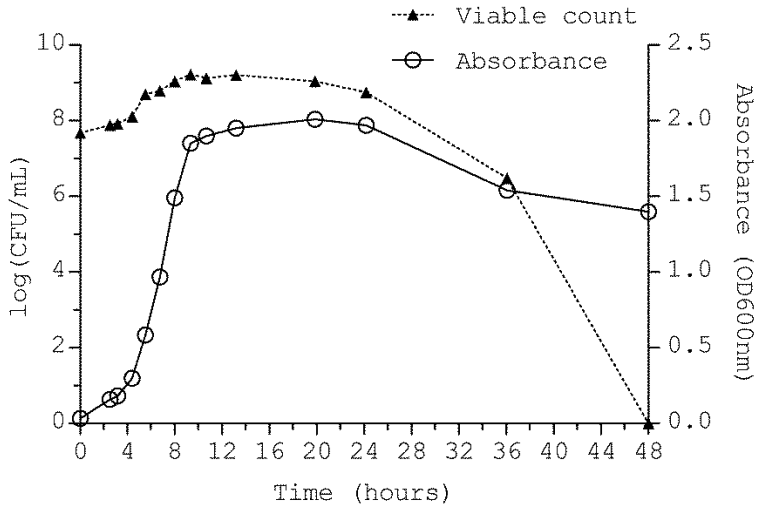

Fig. 2.1. Growth of NIS producing *H. haemolyticus*. Isolate BW1 grown on chocolate agar for 12 hours was used to inoculate 200mL of prewarmed sBHI to an absorbance of 0.03 (OD_{600}), with a viable count of 4.8 x 10^7 CFU/mL, then incubated in a 2L baffled flask for 48 hours at 200 RPM, 37°C for 48 hours.

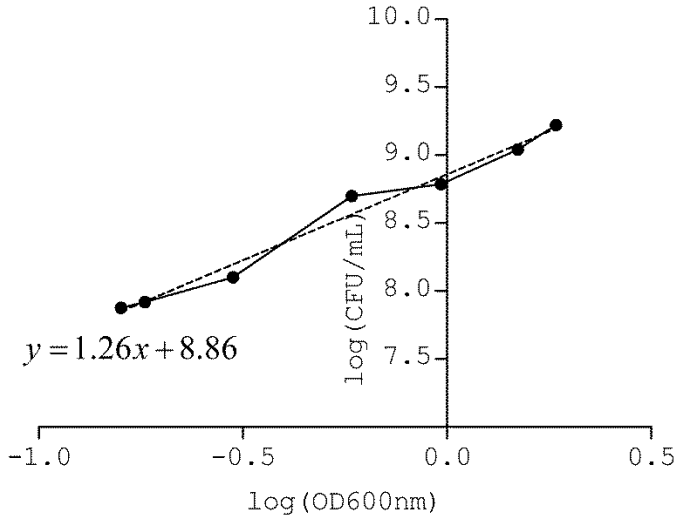

Fig. 2.2. Standard curve for calculating viable count of BW1 from absorbance. NIS-producing *H. haemolyticus* isolate BW1 grown on chocolate agar for 12 hours was used to inoculate 200mL of prewarmed sBHI to an absorbance of 0.03 (OD_{600}), which was also measured at 4.8 x 10^7 CFU/mL, then incubated in a 2L baffled flask for 48 hours at 200 RPM, 37°C. Line of best fit shown for growth phase only, between 2.5 and 9.3 hours.

2.6 Agar well diffusion assay

An agar well diffusion assay was developed for detection and relative quantitation of antimicrobial substances, using a previously described method (Schillinger and Lücke, 1989), but modified for growth of *Haemophilus* spp. The media in the plates and the overlay consisted of 18.5g/L Brain Heart Infusion (Oxoid, CM1135) solidified with 7.5g/L Bacteriological Agar (Oxoid, LP0011) and supplemented with 1% (v/v) resuspended Vitox (Oxoid, SR0090), along with 7.5mg/L each of NAD and haematin (Oxoid, SR0158). These media components are at half the normal concentration. Into 90mm diameter Petri dishes, 10mL of molten media was added with a 25mL serological pipette, and stored at room temperature for 72 hours prior to use or storage at 4°C for up to 8 weeks, prior to use. Overlay media of the same composition was prepared in 30mL aliquots in 50ml "Falcon" centrifuge tubes, and similarly stored before use. To set up an assay, overlay media in 50mL tubes was heated in a 500mL plastic beaker, surrounded by water, in a microwave oven, until fully melted, then transferred to a 45°C water bath for 15 to 60 minutes. To 5mL of this media, 100µL of 1 x 10^6 CFU/mL of indicator strain suspension (see below) was mixed in, then overlayed on a petri dish of solidified media. The plates were then left at room temperature for 30 minutes, then 5mm diameter circular holes were cut in the agar using a sterile stainless steel cork borer. Into the wells were added 25µL aliquots of the test solutions. The plates were then left open in a biological safety cabinet for one hour, at which time there was no longer any free liquid in the wells. Plates were incubated for 18-24 hours, except on the occasions that *H. haemolyticus* was used as an indicator strain, in which case incubation was for 44-48 hours. After incubation, diameters of clearing zones were measured, with results reported in terms of annular radii of clearing zones, which were calculated by subtracting 5mm from the clearing zone diameter, then dividing by 2.

Indicator strains for the agar well diffusion assay were prepared by growing for 6-12 hours on CA then suspending in DPBS to an absorbance of 1.0 (approx. 1 x 10^9 CFU/mL), which was then diluted 10 fold with SMM, and 100µL aliquots stored in microcentrifuge tubes at -80°C. For use, an aliquot was thawed, vortexed briefly, then 5µL mixed with 500µL of DPBS. From this suspension, for each agar well diffusion assay plate, 100µL of this 1 x 10^6 CFU/mL suspension was mixed with 5mL of molten overlay media, as described above. In most cases the indicator strain was NTHi, strain NCTC 4560 and/or NCTC 11315.

In the development of the agar well diffusion assay, we found experimentally that reliable clearing zone formation could only be achieved when assay was set up exactly as described above, with the most important factors being the reduced media concentration and volume, and storage of the media for an extended period. Additionally, the clearing zone size was related to the media age, such that the older the media, the larger the zone size, but also the smaller the size of individual colonies of the indicator strain. As a result of this ageing effect on the media, within-experiment comparison should only be made with media of one age.

2.7 Enrichment of cell-free culture broth

Ammonium sulfate precipitation was used for the enrichment of cell-free culture broth, and was based on the procedure described for haemocin (Venezia et al., 1977). Firstly cell-free culture broth was prepared by centrifugation at 7,000 × g for 15-30 minutes, then ammonium sulfate (Sigma-Aldrich, A-5132) slowly added and dissolved with continuous stirring, to the required % saturation at 4°C (Table 2.1). Solutions were then stored at 4°C for at least two hours, centrifuged at 7,000 × g for 20 minutes, and fractions separated and used as appropriate for the application.

Table 2.1. Ammonium sulfate quantities required to be added to 100mL of solution, for fractional saturation of aqueous solutions, at 4ºC. From http://www.encorbio.com/protocols/AM-SO4.htm.

% Saturation	Ammonium sulfate (g)
10	5.3
20	11.0
30	17.0
40	23.3
50	30.1
55	33.7
60	37.3
70	45.0
80	53.3
90	62.1

2.8 Dialysis

Dialysis in all cases was conducted with 3500 Da molecular weight cut off snakeskin tubing (Thermofisher, 68035). Solutions were dialysed for 24 hours at 4°C against 50mM Tris-HCl pH 7.5, unless otherwise indicated. Mixing was continuous, and the buffer volume was at least 100x the test solution volume, and was changed 3 times in the 24 hours, with at least 2 hours between each buffer change.

2.9 Enrichment by ultrafiltration

Ultrafiltration was conducted to concentrate NIS activity, using a 10 kDA molecular weight cut off centrifugal filter unit (Merck, UFC901024). Centrifugation was at 4000 × g for 15 minutes unless otherwise indicated.

2.10 Extraction of genomic DNA

All isolates were grown overnight on chocolate agar (Oxoid, PP2002) then DNA extracted with a DNeasy Blood & Tissue Kit (Qiagen, 69504) in accordance with kit instructions. The concentration and purity of DNA extracts were assessed with a Nanodrop 1000 spectrophotometer.

2.11 Electrophoresis

PCR products and gDNA were visualised by electrophoresis in 1.5% agarose made up in TAE buffer with SybrSafe. Electrophoresis was for 30 minutes at 100V, and products were visualised on a UV transilluminator.

2.12 SYBR Green Real-time PCR (RTPCR)

Each RTPCR was a total volume 15µL in 0.1mL tubes (Qiagen, 981103), and contained 100pg of template, 333nM each primer (Geneworks, South Australia), and 7.5µL SensiFAST SYBR (Bioline, BIO-98005). Thermocycling (Qiagen, Rotor-Gene Q) consisted of an initial melt at 95°C for 3 minutes followed by 40 cycles of 95°C for 10s, 60°C for 10s and 72°C for 10s. Data was acquired at 72°C, and a melt curve constructed between 60 and 95°C.

2.13 Sanger sequencing

PCR products and plasmids were sequenced at the Australian Genome Research Facility, Melbourne node.

Chapter 3.

Identifying *H. haemolyticus* and *H. influenzae* by SYBR Green real-time PCR assays

This contents of this chapter were submitted for publication in the Journal of Microbiological Methods, as an original research article. It was subsequently revised into a note form, and published in the same journal (original submission 11 December 2014; revision submitted 4 March 2015; accepted 5 March 2015; available online 6 March 2015; volume 112 pp. 67-69; DOI: 10.1016/j.mimet.2015.03.001). This chapter is a compilation of the first and revised submissions, with the formatting adjusted for incorporation into this thesis.

3.1 Introduction

H. influenzae is an opportunistic bacterial pathogen of humans, with the upper respiratory tract its reservoir. *H. haemolyticus* is a bacterium that is also a resident of the upper respiratory tract of healthy people, and has been very occasionally causative of sterile site infections, however in the clinical setting it is generally assumed to be normal flora and non-pathogenic (Murphy et al., 2007). These bacteria are genetically and phenotypically closely related, both being dependent on X and V growth factors. Traditionally these bacteria have been distinguished using conventional culture-based methods, utilizing lysis of horse blood to identify *H. haemolyticus*. However, in a landmark study (Murphy et al., 2007) molecular methods were used to show that up to 40% of conventional culture identified *H. influenzae* isolates from respiratory specimens were in fact *H. haemolyticus.* Other studies have similarly shown this misidentification, albeit at lower rates. As a result, conventional culture is not reliable for identifying these bacteria, and improved methods are required.

DNA-DNA hybridization, and more recently 16S rRNA gene sequencing, are the gold standards for identifying bacteria (Janda and Abbott, 2007; Stackebrandt and Goebel, 1994). However these methods are both time consuming and costly, the former particularly so. Recently, Matrix Assisted Laser Desorption/Ionization (MALDI-TOF) has been implemented in diagnostic laboratories for bacterial identification. Though initially problematic due to inadequate manufacturer databases, differentiating *H. influenzae* and *H. haemolyticus* is now possible with relatively high accuracy (Frickmann et al., 2013; Zhu et al., 2013). The main advantages of MALDI-TOF are that it is rapid and the on-going costs are low, however the initial equipment costs are prohibitively high for many research laboratories.

Optimally, assays for identification of *H. influenzae* and *H. haemolyticus* would be inexpensive, rapid, readily available, and have 100% sensitivity and specificity. PCR-based

assays have the potential to fulfil all these criteria, but none so far have, for these bacteria. Conventional PCR methods (Binks et al., 2012; Norskov-Lauritsen, 2009; Theodore et al., 2012) utilize gel electrophoresis, which is time consuming. Real-time PCR (RTPCR) methods have been developed (Abdeldaim et al., 2009, 2013; Wang et al., 2011) but have so far have been limited by their sensitivity and specificity, and make use of hydrolysis probes which can be costly and problematic to obtain. High resolution melt PCR (HRM-PCR) assays have been developed, and are rapid and inexpensive, but so far they are not sufficiently specific (Binks et al., 2012) or sensitive (Pickering et al., 2014), and we were not able to reproduce the results in our laboratory. SYBR Green RTPCR assays are inexpensive, rapid and readily available, and have been developed for the identification of other bacterial species (Alfaresi and Elkosh, 2006; Clifford et al., 2012; Fukushima et al., 2003), but none so far have been developed for the identification of *H. haemolyticus* and *H. influenzae.*

Gene targets that identify *H. influenzae* and differentiate it from *H. haemolyticus* have been described. Several reports have shown that fuculose kinase (*fucK*) is suitable (Abdeldaim et al., 2009, 2013; Binks et al., 2012; Norskov-Lauritsen, 2009; Theodore et al., 2012) as it is present in most *H. influenzae* isolates but not *H. haemolyticus*. Protein D (*hpd*) is present in both species, but substantial sequence variability between the two has made it possible to design primers and probes specific for *H. influenzae* (Binks et al., 2012; Hare et al., 2012; Pickering et al., 2014; Theodore et al., 2012; Wang et al., 2011).

The number of studies on gene targets for the identification of *H. haemolyticus* is quite limited compared with *H. influenzae*, as there is more research interest in the pathogenic species. Super oxide dismutase C (*sodC*) has been reported as a suitable target (Fung et al., 2006), however there are reports that *sodC* is also present in a minority of NTHi (McCrea et al., 2010b; Norskov-Lauritsen et al., 2009), which may limit its usefulness in this application. In a recent

study (Pickering et al., 2014), *H. haemolyticus hpd* (*Hh-hpd*) was shown useful for its differentiation from NTHi, as it is present in most isolates, and is sufficiently divergent from *H. influenzae hpd (Hi-hpd).*

The aim of this study was to improve upon the methods available in research laboratories to positively identify *H. haemolyticus* and *H. influenzae*. To address this aim we designed and tested specific SYBR Green RTPCR assays for both species, using gene targets *fucK* and *Hi-hpd* for *H. influenzae*, and *sodC* and *Hh-hpd* for *H. haemolyticus*.

3.2 Materials and Methods

3.2.1 Bacterial strains

To assess the performance of the RTPCR assays we tested them against a collection of 127 *H. influenzae* and 60 *H. haemolyticus*. Within this collection, the *H. influenzae* organisms consisted of 119 clinically relevant NTHi isolates, originating from respiratory tract specimens at Australian hospitals, and an additional eight reference strains, ATCC 9007, ATCC 43163, ATCC 10211, ATCC 49766, NCTC 8468, NCTC 4560, NCTC 11315, 86-028 NP. The *H. haemolyticus* organisms consisted of 59 isolates from oropharyngeal swab contamination at Australian hospitals, and ATCC 33390.

The identity of all bacterial isolates was confirmed by sequencing of their 16S rDNA. Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify a 1466bp fragment of the 16S rDNA which was then sequenced with 27F. After manual inspection of chromatograms the sequences were trimmed to 751bp. The species were then determined by comparison with the Genbank database using the blastn algorithm. For this study the search database was restricted to sequences from two publications that included large collections of *H. influenzae* and *H. haemolyticus* (Murphy et al., 2007; Theodore et al., 2012).

3.2.2 Primer Design and SYBR Green real-time PCR

Primers for the RTPCR assays (Table 3.1) were designed using alignments of Genbank nucleotide database sequences for *hpd*, *fucK* and *sodC*. For the *hpd* assays, Genbank was interrogated in March 2014 with search terms "*Haemophilus* AND hpd", with all resulting *H. influenzae* and *H. haemolyticus* sequences aligned, and primers specific for *H. influenzae* (*Hi-hpd*) and *H. haemolyticus* (*Hh-hpd*) selected. For the *H .haemolyticus sodC* specific primers, the same procedure was used, except with search terms "*Haemophilus* AND *sodC*", with primers specific for *H. haemolyticus* selected[1]. Primers for *fucK* were designed using alignments of sequences from a search of Genbank nucleotide database with search terms "*Haemophilus* AND *influenzae* and fucK". The specificity of primers was then verified with blastn searches in Genbank. Conditions for the RTPCR assay are shown in Chapter 2.12.

[1] A minority of non-typeable *H. influenzae sodC* sequences, GU269227, GU269224, GU269216 and GU269206, could not be discriminated from *H. haemolyticus sodC* with this primer set.

Table 3.1 Primers for identification of *H. haemolyticus* and *H. influenzae*.

Name	Sequence, 5' to 3'	Target gene	Amplicon (bp)
sodC F	GTGCGGTATGTTCAGTTG	*H. haemolyticus sodC*	163
sodC R	AGGCCATAGTTAGATTCAGTAAT		
fucK F	TCAATGCTCACTSAACGCTTAAC	*H. influenzae fucK*	150
fucK R	ACGCATAGGAGGGAAATGGT		
Hi-*hpd* F	AAGGGTTATTGGGTAAACTATA	*H. influenzae hpd*	131
Hi-*hpd* R	TCAGGTTTGGATTCTTCTTTATTA		
Hh-*hpd* F	GCGAAAGGTMAATGGGTAAACTAC	*H. haemolyticus hpd*	156
Hh-*hpd* R	TAATGGTGTGTAKACAATGTT		

3.3 Results

The RTPCR results for the 119 NTHi isolates originating from Australian hospitals (Table 3.2) showed that 108 (91%) were positive for both *Hi-hpd* and *fucK*, with the remaining 11 (9%) positive for only *Hi-hpd* or *fucK*. The eight *H. influenzae* reference strains (Table 3.3) were positive for both *Hi-hpd* and *fucK*. No *H. influenzae* were positive for *Hh-hpd* or *sodC*.

For the 59 *H. haemolyticus* isolates from Australian hospitals, the RTPCR results (Table 3.2) showed that all were positive for *Hh-hpd*, and 56 (95%) were also positive for *sodC*. The *H. haemolyticus* strain ATCC 33390 was positive for both *sodC* and *Hh-hpd* (Table 2*)*, and no *H. haemolyticus* organisms were positive for *Hi-hpd* or *fucK*.

Table 3.2. Genotype of NTHi and *H. haemolyticus* originating from Australian hospitals.

No. (%)	***fucK***	***Hi-hpd***	***sodC***	***Hh-hpd***
***A.* NTHi,**				
108 (91)	+	+	-	-
5 (4)	+	-	-	-
6 (5)	-	+	-	-
B. H. haemolyticus				
56 (95)	-	-	+	+
3 (5)	-	-	-	+

Table 3.3. Genotype of reference strains used in this study.

strain species-type	***fucK***	***Hi-hpd***	***sodC***	***Hh-hpd***
ATCC 9007 Hic	+	+	-	-
ATCC 43163 Hib	+	+	-	-
ATCC 10211 Hib	+	+	-	-
NCTC 8468 Hib	+	+	-	-
NCTC 4560 NTHi	+	+	-	-
NCTC 11315 NTHi	+	+	-	-
ATCC 49766 Hib	+	+	-	-
86-028 NP NTHi	+	+	-	-
ATCC 33390 *H. haemolyticus*	-	-	+	+

Sensitivities and specificities for the RTPCR assays were calculated (Table 3.4). All primers were 100% specific, with sensitivities ranging from 95% to 100%. For *H. influenzae* identification, *fucK*, *Hi-hpd*, *fucK* and/or *Hi-hpd*, assay sensitivities were 96%, 95%, 100% respectively, and for *H. haemolyticus*, *Hh-hpd*, and *sodC* assay sensitivities were 100%, and 95%, respectively.

Table 3.4. Sensitivity and specificity of RTPCR assays in this study.

RTPCR assay(s)	sensitivity %	specificity %
fucK[a]	96	100
Hi-hpd[a]	95	100
fucK[a] *and/or Hi-hpd*[a]	100	100
sodC[b]	95	100
Hh-hpd[b]	100	100

[a] specific for *H. influenzae*
[b] specific for *H. haemolyticus*

Analysis of the RTPCR data showed in all cases that a positive reaction resulted in a single melt peak with a cycle threshold between 13 and 18.

3.4 Discussion

The aim of this study was to develop assays for the identification of *H. influenzae* and *H. haemolyticus*, using SYBR Green RTPCR assay technology, as it is a method that is rapid, inexpensive and readily available.

Two primer sets for the identification of each organism were designed, with the assays for *H. influenzae* targeting *fucK* and *Hi-hpd*, and *H. haemolyticus* assays targeting *Hh-hpd* and *sodC*.

Using these assays we were able to correctly identify all the isolates in a collection consisting of 127 *H. influenzae* and 60 *H. haemolyticus* organisms, and as such our results support the use of our SYBR Green RTPCR assays for the identification of *H. haemolyticus* and *H. influenzae* in laboratories in which probe-based RTPCR or MALDI-TOF assays are not available.

The specificities and sensitivities of the assays in our study were broadly consistent with other studies that target the same genes, however there were some inconsistencies. In our study, 95% of the clinically relevant NTHi from Australian hospitals were positive for *fucK* (Table 3.2), exactly matching the proportion reported in a collection of 480 clinically relevant *H. influenzae* (Norskov-Lauritsen, 2009), and close to the 100% reported for 22 strict NTHi carriage isolates (Binks et al., 2012). These proportions are all substantially higher than the 64% reported in a study of NTHi carriage isolates (Theodore et al., 2012), which we suggest may have been due to the use of crude DNA as template in that study.

The results from our RTPCR assays showed that all NTHi in our collection from Australian hospitals were negative for *sodC*. This is consistent with most (Fung et al., 2006; Langford et al., 2002; Norskov-Lauritsen, 2009; Norskov-Lauritsen et al., 2009; Zhang et al., 2014) but not all studies (Langford et al., 1992; McCrea et al., 2010b). However, we had anticipated there would be amplification of *sodC* in the NTHi using our primers, since during the primer design phase we had exploited the reported 5% dissimilarity of *sodC* in *H. haemolyticus* and NTHi (McCrea et al., 2010b). Additionally, our primer design would have excluded amplification of *sodC* in any unexpected isolates of the other known *sodC* positive *H. influenzae*, phylogenetic division II (Kroll et al., 1991) and the cryptic genospecies biotype IV strains (Langford et al., 2002), which we accomplished using the 15% dissimilarity between the *sodC* of *H. haemolyticus* and these *H. influenzae* (McCrea et al., 2010b)

The proportion of *sodC* positive *H. haemolyticus* in our study was 95%, which differs from the 100% reported in the *H. haemolyticus* collections of other studies. However, as the largest of those collections numbers only 110 isolates, and are from a different country, it conceivable our collection had a slightly lesser proportion of *sodC* positive *H. haemolyticus*.

In this study, the SYBR Green *Hi-hpd* RTPCR assay had a sensitivity of 95%, which is within the range of sensitivity values reported elsewhere, 88.5% to 98% (Binks et al., 2012; Theodore et al., 2012; Wang et al., 2011) for the frequently used hpd# 3 hydrolysis-probe RTPCR assay (Wang et al., 2011). The sensitivity of 96% for an HRM-PCR for identification of *H. influenzae* with *hpD* (Pickering et al., 2014) was a remarkably similar value to that of our assay.

Also, our *Hi-hpd* assay was 100% specific, a value higher than reported in most other studies which used *hpd* for identification of *H. influenzae* (Binks et al., 2012; Pickering et al., 2014; Theodore et al., 2012; Wang et al., 2011). We attribute this to primer design, as a larger number of *H. influenzae* and *H. haemolyticus* hpd sequences were available in Genbank at the time we designed our primers. In support of this, the probe-based hpd#3 and hpd#1 RTPCR assays (Wang et al., 2011) had differing specificities when tested on the same isolate collection, with the hpd#1 assay achieving 100% specificity. Interestingly, in that study the hpd#1 was abandoned in favour of the hpd#3 assay, due to it being more suited to use in clinical specimens, as less genome equivalents per reaction were required for a positive reaction. However, in subsequent studies with isolates, hpd#1 has still not been used, in spite of its apparent greater specificity. As are result, we suggest that the use of hpd#1 should be reconsidered for the identification of *H. influenzae* isolates, when hydrolysis probes are preferred over SYBR Green RTPCR assays. .

Relevant to the selection of *hpd* as a target for the detection and identification of *H. influenzae*, is use of PHiD-CV vaccines. These vaccines, which mainly target *Streptococcus pneumoniae* antigens, also contain *H. influenzae* protein D, and there is limited evidence that their use has reduced the incidence of infection due to NTHi (Prymula et al., 2006; Tregnaghi et al., 2014). However, the existence of a subset of disease-associated true NTHi strains that are clearly deficient in *hpd* has been shown (Smith-Vaughan et al., 2014) and while none of these strains were from PHiD-CV vaccinated individuals, a concern remains that continued use of these vaccines may select for these strains. Were such an increase in prevalence of *hpd*-deficient NTHi to occur, any efficacy of the vaccines against NTHi would likely be reduced, as would the usefulness of *hpd* as a target for NTHi identification.

Our *Hh-hpd* assay is the first report we are aware of that uses *hpd* for the identification of *H. haemolyticus*. However it appears the HRM-PCR assay (Pickering et al., 2014) could also be used in this application, but was not reported as such, as the majority of researchers in this field are interested only in the correct identification of *H. influenzae*. However in our laboratory the reported performance of the HRM-PCR was difficult to reproduce, and as such we cannot recommend its use.

The degree to which the results of our study can be extended to *H. influenzae* and *H. haemolyticus* more broadly is limited by the relatively restricted origin of our isolates. Our collection consisted of *H. influenzae* and *H. haemolyticus* isolates from Australian hospitals only. However, that our results were generally consistent with assays that target the same genes, conducted on isolates from other countries (Abdeldaim et al., 2013; Fung et al., 2006; McCrea et al., 2010b; Norskov-Lauritsen, 2009; Theodore et al., 2012; Wang et al., 2011) leads us to the believe that in practice this limitation will be minimal.

This study reinforces the previously reported utility of *hpd*, *fucK* and *sodC* for the identification of *H. influenzae* and its differentiation from *H. haemolyticus*, and improves upon previously described methods, by making use of SYBR Green RTPCR assays , which are relatively rapid, inexpensive and readily available than other published assays commonly used in research laboratories.

Our study is a significant advance for the laboratory identification of *H. haemolyticus*, as it describes sensitive and specific PCR targets. This allows the organism to be positively identified rather than identity being assumed by default based on exclusion of *H. influenzae*, as is often the case.

Identification of an organism as *H. haemolyticus* or *H. influenzae* could in most cases be achieved unambiguously with just two of our SYBR Green RTPCR assays; a single positive RTPCR result for a sequence specific to one organism, accompanied by a single negative result to the other organism. However, as *H. influenzae* and *H. haemolyticus* have very significant genomic heterogeneity, appear to be in an evolutionary continuum (Binks et al., 2012), and can undergo interspecies recombination (Witherden et al., 2014), we suggest the use of all four assays, with 16S rDNA sequencing reserved for ambiguous results. This suggestion of multiple targets is in agreement with other workers in this field that caution no single target is likely to be a perfect diagnostic for either species (de Gier et al., 2016; Pickering et al., 2014; Price et al., 2017).

Chapter 4.

Phenotypic detection of NTHi inhibiting substances (NIS) produced by *H. haemolyticus*

The results in this chapter, for the agar well diffusion assay testing of enriched cell-free culture broths, were incorporated into the publication "An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like substance that inhibits the growth of nontypeable *Haemophilus influenzae*" by Latham, R. D., Gell, D. A., Fairbairn, R. L., Lyons, A. B., Shukla, S. D., Cho, K. Y., . . . Tristram, S. G.' which was published in the International Journal of Antimicrobial Agents in April 2017 (doi:10.1016/j.ijantimicag.2016.12.010).

4.1 Introduction

NTHi, as discussed previously (Chapter 1.2), frequently colonize the upper respiratory tract of healthy children and adults, and are also a major cause of self-limiting opportunistic respiratory infections (Van Eldere et al., 2014). Also, NTHi are increasingly causative of invasive diseases, including septicaemia and meningitis, particularly in infants (Langereis and de Jonge, 2015). With the increasing resistance to antimicrobial therapy, for the management of NTH infections (Dabernat and Delmas, 2012), treatment options are becoming limited. Also, an effective vaccine has yet to be developed (Khan et al., 2016; Teo et al., 2017). In this landscape, alternatives for the prevention and/or treatment should be considered.

Probiotics for the respiratory tract have been developed for the prevention of infections caused by *S. pyogenes* (Di Pierro et al., 2012), *S. pneumoniae* (Marchisio et al., 2015) and *N. meningitidis* (Deasy et al., 2015), but not against NTHi. *H. haemolyticus* is a commensal of the upper respiratory tract, and as such, partially fulfils the requirements as a probiotics against NTHi (Dunne et al., 2001). The potential of *H. haemolyticus* as a probiotic against NTHi was discussed recently in a study which showed *H. haemolyticus* preventing the attachment of NTHi to epithelial cells (Pickering et al., 2016). However, to have probiotic function, strains of *H. haemolyticus* should also secrete NTHi-inhibiting substances (Dunne et al., 2001). But as secretion of an NTHi-inhibiting substance (haemocin) by a closely related organism has already been shown (Venezia,and Robertson, 1975), it is reasonable to suspect that some *H. haemolyticus* may also produce similar substances, and we consider that investigation of this phenomenon is warranted.

In this study, to further investigate the probiotic potential of *H. haemolyticus*, we qualitatively tested *H. haemolyticus* isolates for production of NTHi inhibiting substances (NIS), by screening for inhibitory activity in co-culture and agar well diffusion assays.

4.2 Materials and Methods

4.2.1 *H. haemolyticus* collection

The *H. haemolyticus* collection was composed of 100 randomly selected isolates originating from respiratory specimens submitted to Australian diagnostic laboratories, and throat swabs of healthy volunteers, along with reference strain ATCC 33390. All isolates were identified by 16S sequencing and SYBR Green Real Time PCR for species specific markers, as described in Chapter 3 and elsewhere (Latham et al., 2015). Of these 100 isolates, 61 were randomly chosen for the initial co-culture screening for evidence of inhibitory activity, in which *H. haemolyticus* isolates were pre-incubated prior to addition of *H. influenzae* indicator strains. The second assay, co-culture with simultaneous incubation, tested only the positive isolates from the initial co-culture screening assay. For the third assay, an agar well diffusion assay was used to screen the full collection of 100 isolates for NIS production.

4.2.2 Co-culture assay: Screening pre-incubated *H. haemolyticus* for inhibition of NTHi.

The first method of screening for NIS was a co-culture plate assay, and was a modification of the plate assay method described for the discovery of haemocin (Venezia and Robertson, 1975). Our approach differed in that the test isolates of *H. haemolyticus* were pre-incubated on the plates, and the indicator strains were spot inoculated onto the *H. haemolyticus* colonial growth, rather than being spread over the entire plate. This modification was required because the *H. haemolyticus* growth rate was less than that of *H. influenzae*.

In our assay, the colonial growth of *H. haemolyticus* test isolates were assessed for inhibition of growth of a mixture of *H. influenzae* reference strains. The test isolates were first grown on chocolate agar (CA) then suspended in DPBS to an absorbance of 1.0 (OD_{600}). This suspension was streaked in 5cm lengths on CA plates with a calibrated 10µL plastic inoculation loop, with three streaks to a plate. After incubation of the streaks for 20 hours, a mixed suspension

consisting of equivalent amounts (OD_{600}) of overnight cultures of the *H. influenzae* indicator strains NCTC 11315 (NTHi), ATCC 10211 (Hib), ATCC 43163 (Hib), ATCC 49766 (NTHi), ATCC 9007 (HiC), NCTC 4560 (NTHi), NCTC 8468 (Hib), Rd KW20 (Hid), 86-028NP (NTHi), was prepared in DPBS, to an absorbance of 1.0 (OD_{600}). 10µL aliquots of this suspension were dropped onto the centre of the *H. haemolyticus* colonial growth streaks, with the suspension flowing without aid onto the media adjacent to the colonial growth, in a circular shape of 5 to 10mm diameter. Another 10µL drop of the mixed *H. influenzae* suspension was placed on the plate, but away from the streak, to serve as a growth control. The assay set up is diagrammatically represented below (Fig. 4.1). After incubation for a further 20 hours, *H. influenzae* growth was examined.

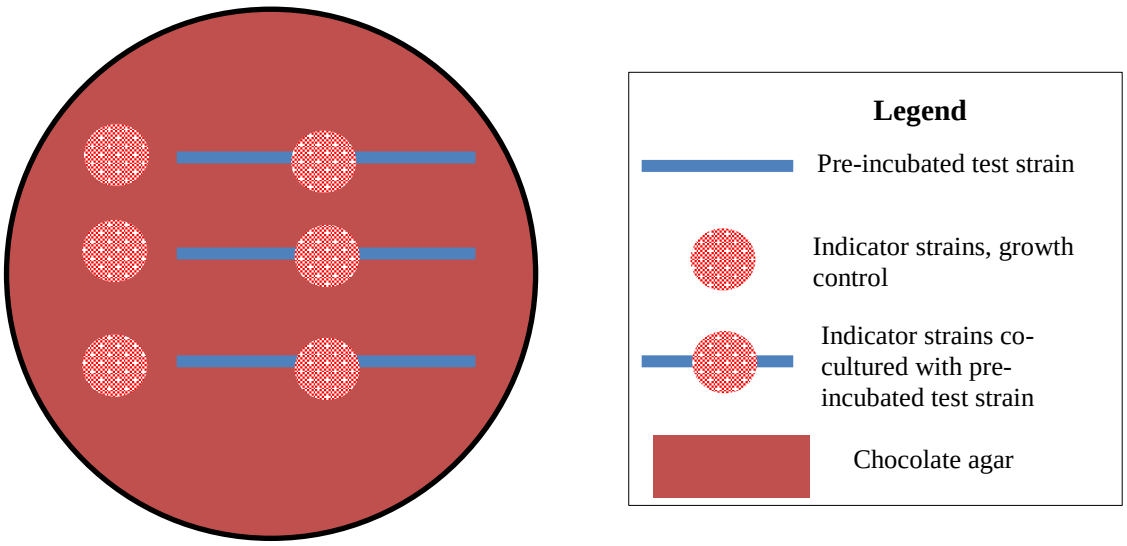

Fig. 4.1. Diagrammatic representation of co-culture assay set up, for the initial screening *H. haemolyticus* isolates for inhibition of NTHi, with test strains pre-incubated. 10µL drops of a suspension of mixed *H. influenzae* indicator strains were placed on the centre of pre-incubated streaks of *H. haemolyticus test strains*. After incubation, *H. influenzae* growth was examined, with inhibitory activity demonstrated by absence of indicator growth on and adjacent to the test strain.

4.2.3 Co-culture assay: NTHi and *H. haemolyticus* incubated simultaneously

The *H. haemolyticus* isolates that were positive for inhibition of the *H. influenzae* growth in the first co-culture assay were tested for inhibition of NTHi using a co-culture assay with simultaneous incubation. The assay was the same as the plate assay method described for the discovery of haemocin (Venezia and Robertson, 1975), but with extended incubation, to allow for the slow growth of *H. haemolyticus*. The indicator strain for this assay was the NTHi reference strain NCTC 11315, which was grown on CA then suspended in DPBS to an absorbance of 0.03 (OD_{600}), with 2.5mL volumes of the suspension spread over plates of sBHI, excess liquid removed with a transfer pipette, and the lids then left open until the surface was dry. For each plate, the colonial growth of the *H. haemolyticus* isolates were stab-inoculated into the sBHI plate with sterile wooden toothpicks, in a grid pattern (Fig. 4.2) with 11mm spacing between each. Twenty-two Hib organisms (MDU isolates 1 to 20, ATCC 8468, ATCC 10211) were included as positive controls, as they are producers of the NTHi inhibiting bacteriocin, haemocin, and the NTHi reference strain NCTC 11315 included as a negative control. The plates were examined for absence of growth of the indicator strain around the test organisms after 19 hours and 43 hours of incubation.

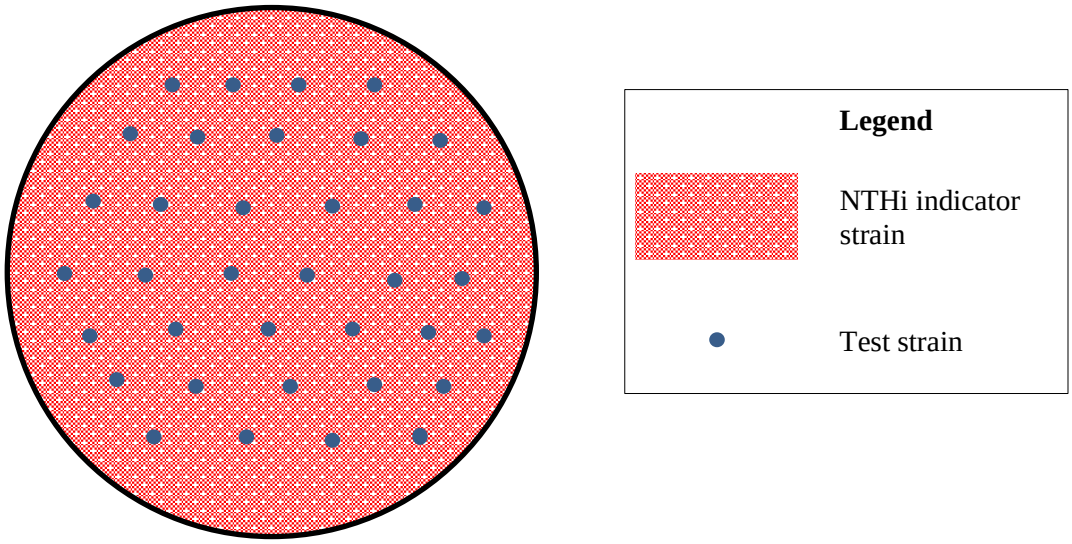

Fig. 4.2. Diagrammatic representation of a co-culture assay, for screening *H. haemolyticus* isolates for inhibition of NTH, with simultaneous incubation. An NTHi indicator strain, NCTC 11315 was spread over over supplemented brain heart infusion agar, with *H. haemolyticus* test strains then stab-inoculated. After incubation, the growth of the indicator strain around the test strains was examined, with absence of growth indicating inhibitory activity.

4.2.4 Screening for NIS production by agar well diffusion assay

An agar well diffusion assay (Chapter 2.6) was developed to further investigate the inhibition of *H. influenzae* that was observed in the initial co-culture screening assay. To obtain a better estimate of the prevalence of NIS production, and to increase the possibility of detecting a rarely produced NIS, the number of isolates screened was increased, from 61 in the first assay, to 100. Each of the wells in the agar contained cell-free culture broth which had been enriched for NIS by ammonium sulfate precipitation to increase the likelihood of detecting inhibitory activity. *H. haemolyticus* isolates which tested positive, as shown by the presence of clearing zones, were re-tested using new preparations of enriched cell-free culture broth, and only isolates that tested positive in both assays are reported as positive for the production of NIS. For comparison purposes, 99 randomly chosen NTHi isolates originating from Australian diagnostic laboratories (Appendix 1.), along with three NTHi reference strains (ATCC 49766, NCTC 4560 and NCTC 11315) were also included in the testing for NIS production.

4.2.5 Preparation of cell-free culture broth

Cell-free culture broths were produced for each isolate, prior to testing in the agar well diffusion assays. Isolates were grown for 6-12 hours on CA, suspended in DPBS to an absorbance of 1.0 (OD_{600}), then 250µL added to 5mL of sBHI in 50-mL non-baffled flasks. These inoculated broths were incubated with 200 RPM agitation for 24 hours at 37˚C. The cultures were then centrifuged at 7000 x g for 30 minutes, and sterile filtered (0.22µm). Cell-free culture broths were tested for NIS activity by agar well diffusion assay (Chapter 2.6), or first enriched by ammonium sulfate precipitation, then tested for NIS activity by agar well diffusion assay. Additionally, enriched cell-free culture broth from the haemocin-producing Hib reference strain ATCC 43163 was prepared as a NIS positive control. Indicators of antimicrobial activity were NTHi reference strains NCTC 11315 and NCTC 4560.

4.2.6 Enrichment of cell-free culture broth by ammonium sulfate precipitation

Cell-free culture broths were enriched for NIS by ammonium sulfate precipitation (Chapter 2.7), with ammonium sulfate added to 55% saturation (Table 2.1), as was used for the discovery of haemocin (Venezia and Robertson, 1975). The resulting ammonium sulfate precipitates were dissolved in a volume of DPBS equal to $1/20^{th}$ the culture broth volume.

4.3 Results

Three assay methods were used to test for inhibition of NTHi by *H. haemolyticus* isolates. Initially, 61 isolates were screened using a co-culture assay, with *H. haemolyticus* pre-incubated before *H. influenzae* were added. In the second assay, six isolates from the first assay that were positive for activity were co-cultured simultaneously with NTHi. For the third assay method, an agar well diffusion assay was used to screen cell-free culture broths from 100 *H. haemolyticus* isolates, for production of NIS.

Using the first co-culture assay, in which 61 pre-incubated *H. haemolyticus* were screened, six isolates (BW1, CF26, L37, L52, L56, M05) inhibited the growth of the mixture of *H. influenzae* reference strains (Table 4.1). The inhibitory activity was observed with naked eye, and seen as an absence of indicator strain growth, adjacent to the pre-incubated *H. haemolyticus* streaks (Fig. 4.3A). The other *H. haemolyticus* organisms did not inhibit the indicator strains, such that they grew right up to, and in some cases, over the test strains (Fig. 4.3B, 4.3C). The six isolates testing positive for inhibition of *H. influenzae* were then subjected to further testing in a simultaneous incubation co-culture assay. However, they did not inhibit the growth of NTHi (Fig. 4.4A.), while the positive control Hib organisms produces small zones of inhibition (Fig. 4.4B), extending approximately 1mm out from the colonial growth.

The positive results for the agar well diffusion assay depended on enrichment of activity in the cell-free culture broth. No unenriched cell-free culture broth preparations, including the positive control Hib strain, inhibited the NTHi indicator strains. Whereas with the use of enriched cell-free culture broth, the two *H. haemolyticus* isolates BW1 and RHH122 consistently inhibited both indicator strains (Fig. 4.5, only BW1 inhibition of NCTC 4560 is shown), while the enriched cell-free culture broth preparations from the other 98 *H. haemolyticus* isolates (Table 4.1), and the 102 NTHi organisms included for comparison purposes (Appendix 1.), were negative.

Table 4.1. *H. haemolyticus* isolates inhibiting NTHi. The listed *H. haemolyticus* were screened for inhibition of NTHi by co-culture and/or agar well diffusion assay. For co-culture, isolates were pre-incubated on chocolate agar prior to spot inoculation with a suspension of mixed *H. influenzae* reference strains, then re-incubated. For agar well diffusion assay, cell-free culture broths were enriched by ammonium sulfate precipitation, then tested against NTHi reference strains NCTC 4560 and NCTC 11315. Left symbol: result for screening by co-culture assay, right symbol: result for screening by agar well diffusion assay, – : negative, + : positive, o : not tested, bold : a positive result in either assay.

ATCC 33390 - -	CF69 - -	L152 o -	RHH11 - -
		L153 o -	RHH13 - -
BW1 + +	L3 o -	L187 o -	RHH16 - -
BW2 - -	L4 - -	L190 o -	RHH40 - -
BW5 - -	L5 - -	L191 o -	RHH57 - -
BW6 - o	L6 - -	L274 o -	RHH58 - -
BW15 - -	L7 - -	L292 o -	RHH59 - -
BW16 o -	L8 o -	L320 o -	RHH61 - -
BW18 - -	L12 - -	L345 o -	RHH65 - -
BW33 o -	L16 o -		RHH67 - o
BW34 o -	L19 - -	**M05 + -**	RHH75 - -
BW36 o -	L23 - -	M16 o -	RHH112 - -
BW39 o -	L34 o -	M22 - -	**RHH122 - +**
	L37 + -	M26 - -	
BWOCT 2 o -	L42 o -	M28 o -	UTAS 161 - o
BWOCT 3 o -	L43 - -	M30 o -	
BWOCT 9 o -	L47 o -	M34 - -	
BWOCT 12 o -	L48 - -		
BWOCT 13 o -	L50 o -	NF1 - -	
BWOCT 14 o -	L51 o -	NF2 o -	
BWOCT 16 o -	**L52 + -**	NF4 - -	
BWOCT 17 o -	L54 o -	NF5 - -	
BWOCT 18 o -	L55 o -	NF6 - -	
BWOCT 20 o -	**L56 + -**	NF11 - -	
	L61 o -	NF14 - -	
CF5 - -	L66 - -	NF15 - -	
CF14 - -	L71 - -	NF19 - -	
CF25 - -	L72 o -	NF22 - -	
CF26 + -	L73 o -	NF28 - -	
CF28B - -	L93 - -	NF34 - -	
CF53 - -	L95 - -	NF36 - -	
CF65 - -	L117 o -		

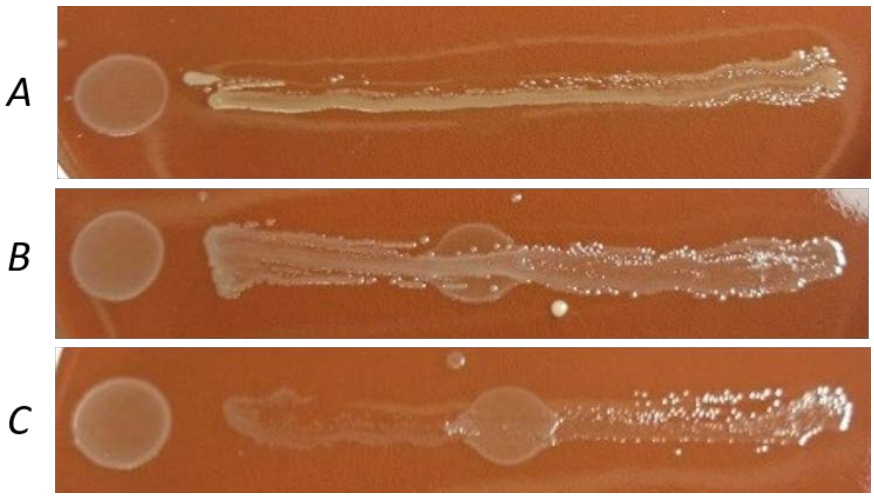

Figure 4.3. Inhibition of *H. influenzae* by pre-incubated *H. haemolyticus*. 10µL droplets of mixed *H. influenzae* suspension were placed on the the middle of preincubated *H. haemolyticus* streaks, and separately (circular colony to the left of the streak) for a growth control, on chocolate agar. After incubation, the growth of *H. influenzae* was assessed visually, with absence of growth adjacent to the streak (A) indicating inhibition, and growth on and adjacent to the streaks (B, C) indicating no inhibition.

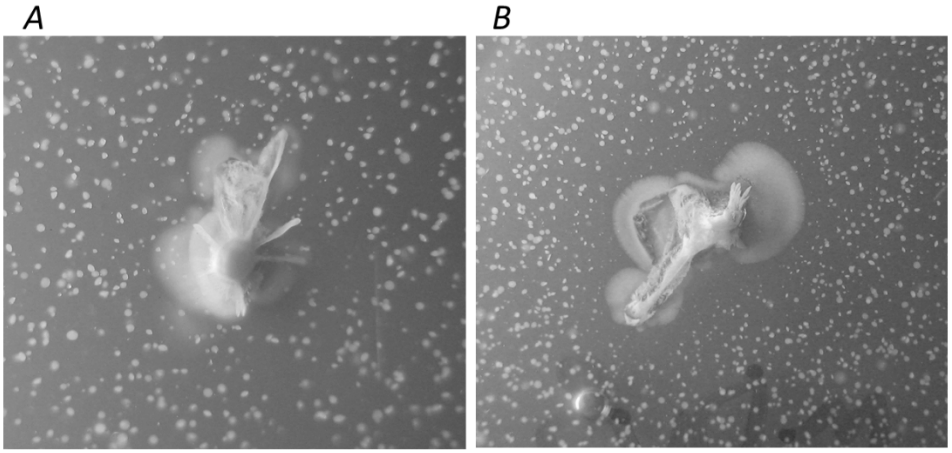

Figure 4.4. Inhibition of NTHi by Hib but not *H. haemolyticus*, in simultaneous co-culture. The growth of NTHi indicator strain NCTC 11315, spread over sBHI agar, was inhibited by all simultaneously grown Hib organisms, including MDU 3 (B) but not by the *Haemophilus haemolyticus* isolate BW1 (A) or any other *H. haemolyticus* test isolates.

Fig. 4.5. Inhibition of NTHi by *H. haemolyticus* BW1. Growth of NTHi reference strain NCTC 4560 is inhibited around a well containing enriched cell-free culture broth from *H. haemolyticus* isolate BW1. + is enriched cell-free culture broth from *H. influenzae* type b reference strain ATCC 43163, a positive control for the assay, as it produces haemocin. – is enriched cell-free culture broth from negative control NTHi reference strain NCTC 4560.

4.4 Discussion

This study was a preliminary investigation of the potential of *H. haemolyticus* in the respiratory tract to reduce NTHi infection, with the assays intended to show phenotypical evidence for inhibition of NTHi growth, as well as NIS production. Using co-culture plate and agar well diffusion assays, there was evidence that a minority of *H. haemolyticus* isolates were able to inhibit NTHi, and produce a NIS. In our first co-culture assay, the colonial growth of 6 of 61 pre-incubated *H. haemolyticus* inhibited a mixed culture of *H. influenzae*. However, when these six isolates were incubated in co-culture simultaneously with NTHi, no inhibitory activity was observed. To further investigate the inhibitory activity observed in the first co-culture assay, an agar well diffusion assay was developed to test for NIS in cell-free culture broth from the six isolates. Yet, it was only when the cell-free culture broth was enriched for NIS by ammonium sulfate precipitation was inhibitory activity observed, and only by a single isolate, BW1. Then, using the agar well diffusion assay for screening a larger collection of *H.*

haemolyticus for NIS, we were able to detect one other NIS producer, RHH122, and thus the prevalence of NIS production in our *H. haemolyticus* collection was 2 in 100 (2%).

Co-culture assays on solidified media, as used in this study, have been used extensively for the screening of microorganisms including, *Haemophilus* spp., for production of antimicrobial substances (LiPuma et al., 1990, 1992, Murley et al., 1997, 1998; Venezia and Robertson, 1975). The advantages of such assays are rapidity and cost effectiveness, and their potential to induce the production of antimicrobial substances which would not be produced in monoculture (Maldonado-Barragán et al., 2013). In our study, using a co-culture assay in which *H. haemolyticus* test isolates were pre-incubated, 6 of 61 isolates inhibited *H. influenzae*. However, when the inhibiting isolates were tested in a second co-culture assay, which utilised simultaneous incubation, the activity was no longer apparent. These observations suggest that a large established *H. haemolyticus* population is required for an inhibitory factor to be produced, and as well demonstrate a weakness of the co-culture assay technique - that they convey scarce information about the environmental requirements for production of inhibitory activity, or the mechanism of the inhibition. Indeed, the observed inhibitory activity in co-culture could be due to non-specific mechanisms, such as depletion of nutrients or excretion of metabolites. However we consider such a non-specific mechanism unlikely, because only 10% of *H. haemolyticus* inhibited *H. influenzae*. Nevertheless, we developed the third assay method, an agar well diffusion assay, to further investigate the inhibitory activity observed in the first assay, and to help establish that NIS was being produced.

Agar well diffusion assays, as used in this study, are another technique that has been used extensively in the investigation of antimicrobial substances, including those produced by *Haemophilus* spp. (He et al., 2006; Schillinger and Lücke, 1989; Streker et al., 1981; Venezia and Robertson, 1975; Venezia et al., 1977). A strength of the technique is that it more

effectively identifies inhibition effects due to substances produced by organisms, from the effects due to depletion of nutrients in the growth media that accompany growth. However, as well as being substantially more time consuming, a weakness of this and other indirect techniques is that inhibitory substances are being tested in a different environment from which they were produced, and as a result do not produce the localised concentration spikes that occur when substances are produced *in situ*. Indeed, as was the case in the discovery of haemocin (Venezia and Robertson, 1975), no inhibitory activity could be observed until the cell-free culture broth was concentrated 20-fold by ammonium sulfate precipitation. This reduced concentration effect may also help explain the difference in estimated prevalence of inhibitory activity observed between the assays; 10% for co-culture, and 2% for the agar well diffusion assay.

In this study, there were substantial differences in the results between the two screening assays for inhibitory activity. The differences in prevalence are discussed above, but, more puzzling is that the apparently more sensitive initial co-culture screening assays showed RHH 122 did not produce NIS, but the agar well diffusion assay screening did. At this stage we do not have enough experimental evidence regarding the mechanism of inhibition in either the co-culture or agar well diffusion assay for us to explain these differences. However, although these differences are informative, and important for future studies in which isolate collections are screened for inhibitory activity, it will likely be a moot point for this project, as subsequent research will be dominated by testing of enriched cell-free culture broth preparations, and only the agar well diffusion assay will be used.

Our results are the first we are aware of that show *H. haemolyticus* can inhibit the growth of other bacterial species. However, *Haemophilus* spp. have previously been shown to inhibit the growth of bacterial species, with Hib the archetype, as they produce haemocin (Venezia and

Robertson, 1975), a bacteriocin which inhibits NTHi. Interestingly, *H. influenzae* and *H. haemolyticus* appear to be in an evolutionary continuum (Binks et al., 2012) and can undergo interspecies recombination (Witherden et al., 2014), which presents the possibility that haemocin genes may be present in both species, and that the observed inhibitory activity of *H. haemolyticus* isolates, was due to production of haemocin. Indeed, as was the case with haemocin (Venezia and Robertson, 1975)it was only when the cell-free culture broth was concentrated 20-fold by precipitation with 55% saturated ammonium sulfate was inhibitory activity detected in the agar well diffusion assay. Even though genetic testing is the preferred method of testing, for the following two reasons we suggest our results so far show that our *H. haemolyticus* isolates are unlikely to be producing haemocin. Firstly, in the co-culture screening assay, six *H. haemolyticus* isolates inhibited a mixed culture of *H. influenzae*, which included four Hib strains – an unlikely result if the six *H. haemolyticus* were producing haemocin, as the great majority of Hib are also producers of haemocin (LiPuma et al., 1990) and have genes for haemocin immunity (Murley et al., 1997). Secondly, in the simultaneous co-culture plate assays, the Hib isolates produced clearing zones, which any haemocin producing *H. haemolyticus* would also be expected to do, but they did not.

In this study, we observed the inhibition of *H. influenzae,* including NTHi, by a minority of *H. haemolyticus* organisms. This inhibitory activity appeared to be due the movement of inhibitory substance(s) through the growth media. However, we consider it unlikely that this substance was haemocin, the bacteriocin produced by Hib. As such our results broadly support the idea that some *H. haemolyticus* organisms exist which could be used as probiotics to inhibit NTHi, as has been suggested previously (Pickering et al., 2016). Nevertheless, at this stage the generalizability of the main findings from this study are substantially limited by their *in vitro* nature, and further support for the use of such organisms as probiotics would require that this

inhibitory activity still exist in the complex microbial environment of the human respiratory tract. To this end, we recommend further studies of the characteristics, structure, production and mechanism of action of the NIS produced by *H. haemolyticus*, and although so far we have compared the inhibitory activity of *H. haemolyticus* to that of the bacteriocin haemocin, it is important to recognise there are other types of diffusible substances that specifically inhibit growth of microbes, including siderophore and quorum sensing substances (Hibbing et al., 2010).

Chapter 5.

Characteristics of an NTHi inhibiting substance produced by *H. haemolyticus*

The results in this chapter were incorporated into the publication "An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like substance that inhibits the growth of nontypeable *Haemophilus influenzae*" by Latham, R. D., Gell, D. A., Fairbairn, R. L., Lyons, A. B., Shukla, S. D., Cho, K. Y., . . . Tristram, S. G.' which was published in the International Journal of Antimicrobial Agents in April 2017 (doi:10.1016/j.ijantimicag.2016.12.010).

5.1 Introduction

In Chapter 4, we showed evidence for production by *H. haemolyticus* of a diffusible substance which inhibited the growth of NTHi. Using a co-culture assay, 6 of 61 *H. haemolyticus* isolates (BW1, CF26, L37, L52, L56, M05) inhibited a mixed culture of *H. influenzae,* including NTHi. Then, using an agar well diffusion assay, growth of NTHi was inhibited by the enriched cell-free culture broth from the *H. haemolyticus* isolates, BW1 and RHH122. For the development of *H. haemolyticus* as a probiotic, further characterisation of the NIS produced by *H. haemolyticus* was required.

In this chapter, we sought to understand the chemical and physical properties of the NIS produced by *H. haemolyticus* BW1 (BW1-NIS). This isolate was selected for further study as it was the only *H. haemolyticus* isolate which showed activity in both the co-culture and agar well diffusion assays. Similar to previous studies identifying novel antimicrobial substances (He et al., 2006; Venezia and Robertson, 1975), we examined the ammonium sulfate concentration required for enrichment, production in relation to bacterial growth, spectrum of activity, resistance to inactivation by heat, resistance to inactivation by extremes of pH, resistance to degradation by enzymes, and apparent molecular size. Additionally, BW1 gDNA was tested for presence of the structural gene for haemocin, *hmcA*.

5.2 Materials and Methods

5.2.1 Assessment of inhibitory activity

Agar well diffusion assays (Chapter 2.6) were used for the assessment of NIS activity in enriched cell-free culture broth (Chapter 2.7), with the NTHi reference strain NCTC 4560 as the indicator.

5.2.2 Preparation of NIS produced by BW1 (BW1-NIS)

Cell-free culture broth from *H. haemolyticus* isolate BW1 was enriched for NIS and used for the experiments in this study. Cultures of BW1 were grown on CA for 12-16 hours (except in the case of production with time and population experiments), then suspended in pre-warmed (37˚C) sBHI to an absorbance of 0.05 (OD_{600}). Inoculated broths were then incubated with 200 RPM agitation for 24 hours at 37˚C, and supernatants collected after centrifugation at 7000 x g, 30 minutes. Cell-free culture broths were then enriched for NIS (Chapter 2), with ammonium sulfate added at 70%, a value chosen from the ammonium sulfate concentration optimisation experiments in this Chapter. The precipitates were dissolved in a volume of DPBS equal to $1/20^{th}$ of the initial culture broth volume, heated at 90˚C for 3 minutes (except in the case of testing for resistance to inactivation by heating) for microbial decontamination and denaturing of heat labile proteins, then centrifuged at 10,000 × g for 10 minutes, and supernatants collected for characterisation experiments. Preparations for chromatographic separations and inactivation experiments were also dialysed (Chapter 2.8), concentrated to the pre-dialysis volume by ultracentrifugation (Chapter 2.9), and finally passed through a 0.2 μm filter. Haemocin was also prepared, from Hib reference strain ATCC 43163, as described previously (Chapter 4.2.5).

5.2.3 Ammonium sulfate concentration optimisation

Variable amounts of ammonium sulfate were added to a single batch of BW1 cell-free culture broth to determine the concentration required for BW1-NIS precipitation. Ammonium sulfate was added to 10mL aliquots of cell-free culture broth in 50mL centrifuge tubes, to achieve saturations of ranging from 30% to 80% (Table 2.1), dissolved immediately by inversion, and processed as described for enrichment of cell-free culture broth (Chapter 2.7). The resulting precipitates were dissolved in DPBS as described above, and tested for activity. A separate experiment was conducted, to determine if additional activity was recoverable from a 55%

saturated solution, as used for enrichment in Chapter 4, by addition of ammonium sulfate to 70% saturation.

5.2.4 Production of NIS by BW1

For assessment of the growth stage at which BW1-NIS was being produced, 5 x 400mL volumes of sBHI broths were prepared in 2000-mL baffled flasks, inoculated as described above, but with BW1 inoculum culture grown on CA for 10 hours, and a broth incubation time of 12 hours. At regular intervals the five flasks were sampled for measurement of absorbance (OD_{600}), and assessment of inhibitory activity. Viable counts were calculated from absorbances, using an equation from a standard curve (Chapter 2.5.3, Fig. 2.2.).

5.2.5 Spectrum of activity

Agar well diffusion assays (Chapter 2.6) were used to determine the activity of BW1-NIS against a range of clinically relevant bacteria. Assays were set up with overlayed indicator strains variably composed of NTHi (n = 102), Hib (n = 21), *H. haemolyticus* (n = 30), *Acinetobacter baumanii* (n = 1), *Burkholderia cepacia* (n = 1), *Enterococcus* spp. (n = 4), *Escherichia coli* (n = 3), *H. parainfluenzae* (n = 2), *H. influenzae* type c (n = 1), *Moraxella catarrhalis* (n = 1), *Neisseria lactamica* (n = 1), *Proteus vulgaris* (n = 1), *Pseudomonas aeruginosa* (n = 1), *Staphylococcus* spp. (n = 4), and *Streptococcus* spp. (n = 5). For comparison purposes, the spectrum of activity of a haemocin preparation from Hib reference strain ATCC 43163 was determined with the same *H. haemolyticus* collection (n = 30).

5.2.6 Resistance to inactivation

The chemical stability of the active component in BW1-NIS was examined by assessing its resistance to conditions which degrade proteins; heat, pH extremes and proteinases. Also, as for the original description for haemocin (Venezia and Robertson, 1975), the possibility that the antimicrobial activity was due to a bacteriophage was examine, by treatment of BW1-NIS

with DNase. For assessment of resistance to heat, three experiments were undertaken. In the first experiment, 250µL aliquots of BW1-NIS in pre-heated 1500 µL microcentrifuge tubes were incubated for 15 minutes in a heating block at set temperatures of 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C, centrifuged at 16,000 x g for 10 minutes, to pellet the resulting suspending precipitates, and supernatants tested for activity. In the second heating experiment, 100 µL aliquots of BW1-NIS in 1500 µL microcentrifuge tubes were heated for 2 hours in a dry heating block set to 95°C, with actual solution temperatures continuously measured using a thermometer in a blank tube, which contained 100µL of water. Tubes were removed from the heating block at intervals of 7, 10, 20, 30, 60 and 120 minutes from commencement of incubation. For the third heating experiment, 4 x 40µL aliquots of BW1-NIS were autoclaved in plastic 200µL PCR tubes at 110°C for 15 minutes. Resistance of 200µL aliquots of BW1-NIS to inactivation by pH extremes was determined by adjusting the pH to values of 1.6, 2.1, 3.3, 4.5, 5.3, 6.4, 7.2, 7.9, 8.6, 9.1, and 9.7 with a calibrated ISFET pH meter (Shindengen, IQ25 Mini) and 0.1M and 1.0M solutions of HCl and NaOH, incubating for 1 hour at room temperature, neutralizing back to pH 7.2, then equalizing the volumes with DPBS. Resistance to enzymatic degradation was determined by addition of enzymes to a final concentration of 1 mg/mL, using proteinase K (Bioline, BIO-3708), 1 mg/mL Trypsin (Sigma-Aldrich, T4049) and 100U/mL DNAse I (Invitrogen, 18068015). The enzyme containing solutions were incubated at 37°C for 20 hours, heated at 90°C for 3 minutes for microbial decontamination, centrifuged at 16,000 x g for 10 minutes, and supernatants tested for activity.

5.2.7 Molecular size

The molecular size of the active component in BW1-NIS was estimated by size exclusion chromatography (SEC). BW1-NIS was fractionated with a calibrated HiLoad 26/600 Superdex 200 column (GE Healthcare Life Sciences) with a bed volume of 320mL and a sample load of

10mL, equilibrated in 0.15M sodium phosphate buffer, pH 7.0. The column was calibrated with native globular proteins, thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa), as standards (Bio-Rad, Hercules).

5.2.8 Real-time PCR for haemocin

DNA was extracted from BW1, and tested for the presence of the Hib haemocin structural gene (*hmcA*) by RTPCR (Chapter 2.12) of a 151bp fragment with primers 2533F (5'-CAGCTTCGCTAGCAAGTAGTAATG-3') and 2683R (5'-TGTTTTGCTCCGCATATTGA-3') designed from the Hib haemocin locus (accession no. U68399).

5.3 Results

Ammonium sulfate precipitation is a method that is commonly used to selectively enrich peptides or proteins from complex and dilute mixtures, early in a purification procedure. Two experiments were set up to determine the ammonium sulfate concentration required to enrich BW1-NIS in cell-free culture broth. In the first, no activity was detectable at saturations of 30% and 40%, inhibitory activity was detectable at 50% saturation, and the maximum activity was recovered at saturations of 60%, 70% and 80% (Fig. 5.1). In the second experiment, working with a 55% saturated solution, addition of ammonium sulfate to 70% saturation resulted in the recovery of a similar amount of activity to that which was recovered from the 55% saturation precipitate. As a result of these two experiments, and to maximise recovery, a saturation of 70% was used for enrichment of BW1-NIS in all other experiments in this chapter.

The production of BW1-NIS was measured over a 12 hour period of broth culture (Fig. 5.2). NIS activity was first detected late at 7 hours of incubation, at which time the bacteria were in late growth phase, the absorbance (OD_{600}) was 1.5, and the viable count was 1.2×10^9 CFU/mL.

The activity continued to increase after stationary phase was attained at 10 hours, at which time the absorbance (OD_{600}) was 2.0 and the viable count was 1.7 x 10^9 CFU/mL.

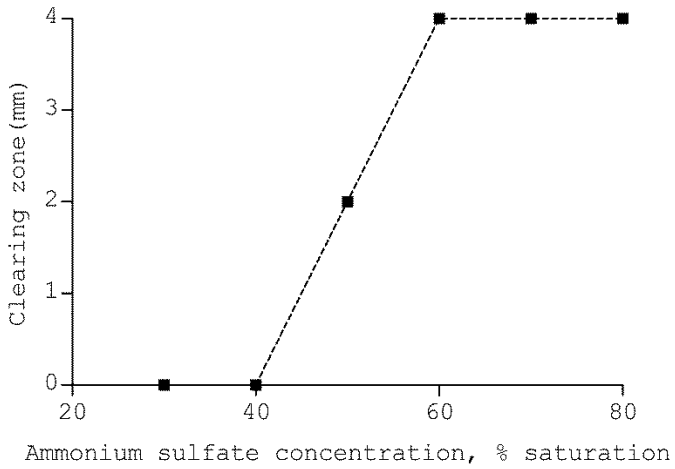

Fig. 5.1. Enrichment of an NTHi inhibiting substance (NIS) in *H. haemolyticus* BW1 cell-free culture broth by ammonium sulfate precipitation. At 50 % saturation the NIS was partially extracted, but at least 60 % was required for full extraction. Ammonium sulfate solutions were incubated for 2 hours at 4°C, centrifuged, then precipitates resuspended in 1/20th volume of DPBS. Activity assessed by agar well diffusion assay with NTHi indicator strain NCTC 4560 (annular radius of clearing zones).

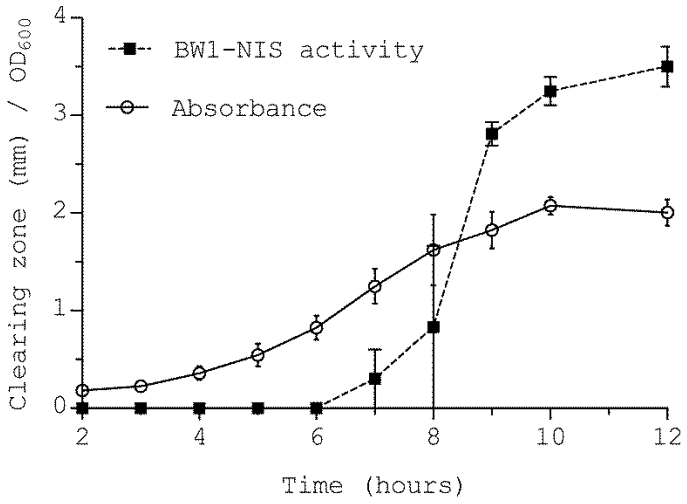

Fig. 5.2. Production of BW1-NIS in broth culture. The BW1-NIS activity was first measurable at an OD_{600} of 1.5 at 7 hours of incubation, and continued to increase until after stationary phase was attained at 10 hours. *H. haemolyticus* BW1 grown in sBHI broth at 37˚C with 200 RPM agitation. Activity assessed by agar well diffusion assay against NTHi indicator strain NCTC 4560. Annular radius of clearing zone, mean +/- SEM, five independent experiments.

The spectrum of activity of BW1-NIS was tested by agar well diffusion assay, with NTHi and other clinically relevant bacteria being the overlayed indicator strains in the assays. All of 102 NTHi, 21 Hib, and 17 of 30 *H. haemolyticus* were inhibited by BW1-NIS. The *H. haemolyticus* strains BW1, ATCC 33390 and the other 11 isolates were not inhibited. No other bacteria were inhibited by BW1-NIS (Table 5.1). The complete spectrum of activity testing results for BW1-NIS are shown in Appendix 2.

Table 5.1. Spectrum of activity of BW1-NIS. The activity of a NIS produced by *H. haemolyticus* BW1 was tested against listed organisms, in agar well diffusion assays. Complete results are in Appendix 2.

Genus species, capsular serotype	**Origin**	**No. inhibited/ No. tested (%)**
Haemophilus influenzae, NTHi	NCTC 4560, NCTC 11315, isolates	102/102 (100)
Haemophilus influenzae, b	ATCC 43163, isolates	21/21 (100)
Haemophilus influenzae, c	ATCC 9007	1/1
Haemophilus haemolyticus	ATCC 33390, isolates	17/30 (57)
Haemophilus parainfluenzae	Isolates	0/2
Acinetobacter baumanii	ATCC 17409	0/1
Burkholderia cepacia	ATCC 25416	0/1
Enterococcus faecalis	ATCC 29212, POW 1994	0/2
Enterococcus faecicum	Isolate	0/1
Enterococcus gallinarum	ATCC 49573	0/1
Escherichia coli	NCTC 11560, ATCC 10418, DH5α	0/3
Moraxella catarrhalis	ATCC 25238	0/1
Neisseria lactamica	ATCC 23970	0/1
Proteus vulgaris	ATCC 8427	0/1
Pseudomonas aeruginosa	ATCC BAA-47	0/1
Staphylococcus aureus	ATCC 25923, 29213, 33591	0/3
Staphylococcus epidermidis	ATCC 12228	0/1
Streptococcus bovis	ATCC 35034	0/1
Streptococcus pneumoniae	NCTC ARL 10582, ATCC 6305	0/2
Streptococcus pyogenes	ATCC 19615	0/1

In a comparison of the spectrum of activity of haemocin and BW1-NIS against the 30 *H. haemolyticus* isolates, the results were highly variable. Although a regression of clearing zones size showed a significant positive relationship overall (Fig. 5.3), they could be divided into four groups. Twelve isolates were inhibited by neither, 3 were inhibited by haemocin only, 4 were inhibited by BW1-NIS only, and 11 were inhibited by both. The complete spectrum of activity testing results are shown in Appendix 2.

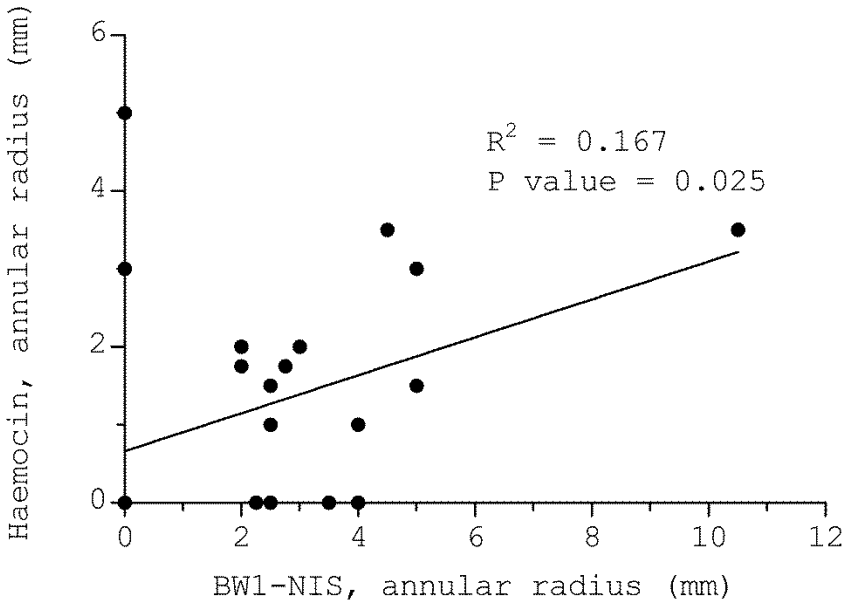

Fig. 5.3. The inhibition of *H. haemolyticus* growth, by haemocin and BW1-NIS, are positively related. Using agar well diffusion assays, preparations of haemocin and BW1-NIS were tested against *H. haemolyticus* organisms (n = 30), and clearing zones measured. Markers at (0,0) and (0, 3) comprised of 12 and 2 isolates respectively. Complete results are in Appendix 2.

The resistance of BW1-NIS to heating was assessed in three experiments of varying incubation temperature and time. In the first heating experiment, there was no reduction in activity following incubation of samples for 15 minutes in a heating block which was set to temperatures ranging from 40 to 100°C. In the second experiment, the blank attained 90°C after 7 minutes of heating, and the activity was undiminished at 30 minutes, at 60 minutes and 120 minutes (Fig. 5.4). For the third experiment, no activity was detectable after autoclaving. In testing of the resistance of BW1-NIS to inactivation by pH extremes, the activity was undiminished, when incubated at room temperature for 1 hour, at pH values from 1.6 to 9.7 (Fig. 5.5).

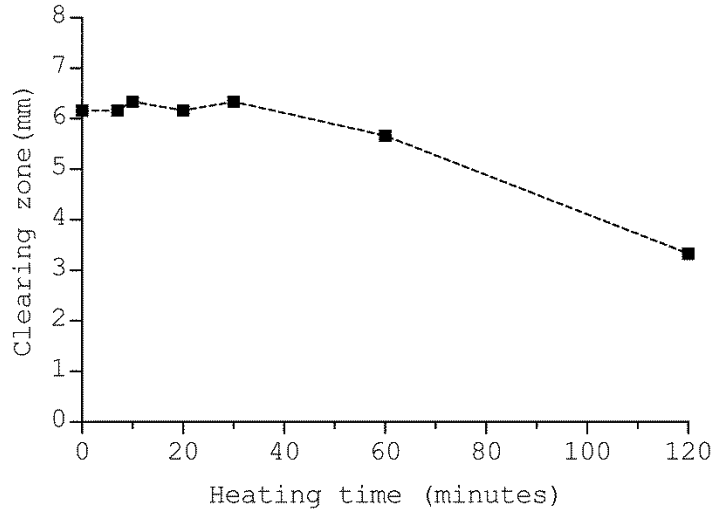

Figure 5.4. Stability of BW1-NIS at 90°C. Activity assessed by agar well diffusion assay against NTHi indicator strain NCTC 4560, with annular radius of clearing zones shown.

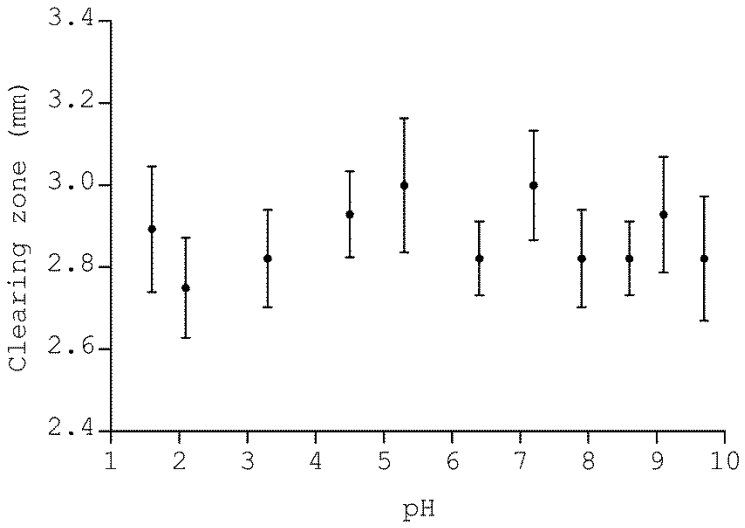

Figure 5.5. Stability BW1-NIS at pH extremes. A preparation of BW1-NIS was adjusted to varying pHs with HCl and NaOH, incubated for 1 hour at room temperature, then neutralized. Three replicates per treatment. Activity measured by agar well diffusion assay with NTHi indicator strain NCTC 4560 (annular radius of clearing zones).

The chemical composition of the active component in BW1-NIS was assessed, by treatment with proteases and a DNAse. After incubation overnight at 37°C, proteinase K reduced BW1-NIS activity to levels that were undetectable, whereas trypsin treatment reduced the activity but it was still detectable. Treatment with DNAse I did not reduce the activity.

The molecular size of BW1-NIS was determined using size exclusion chromatography. A 10mL load of BW1-NIS was passed over a Superdex 200 column, and 69 x 4mL samples collected, with activity detected in fractions 33 to 41, with the peak of activity in fraction 37, at an elution volume of 222mL (Fig. 5.6). The molecular size of BW1-NIS was then predicted, with a globular protein molecular weight standard, to be approximately 32 kDa, under native conditions (Fig. 5.7).

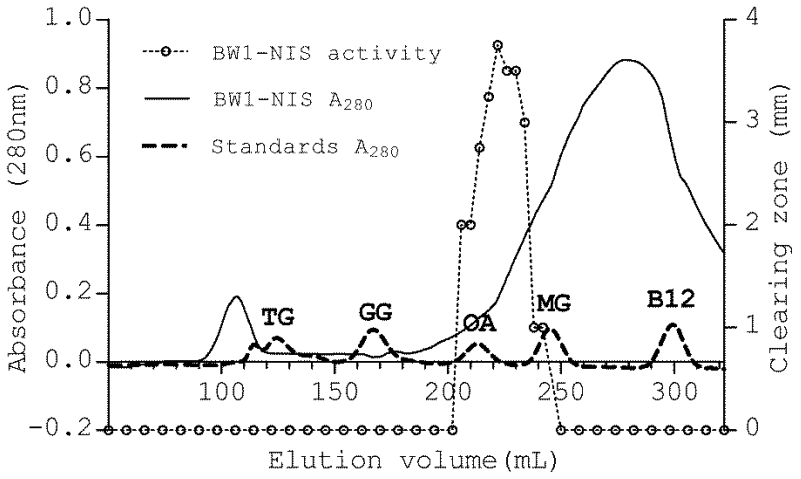

Fig. 5.6. Size exclusion chromatography of BW1-NIS. The peak of BW1-NIS activity occurs in the fraction with an elution volume of 222mL, between the volumes at which ovalbumin and myoglobin are eluted from the column. Activity assessed by well diffusion assay against NTHi indicator strain NCTC 4560 (annular radius of clearing zone). Molecular weight standards thyroglobulin (TG), γ-globulin (GG), ovalbumin (OA), myoglobin (MG). Vitamin B12 (B12) indicates buffer front.

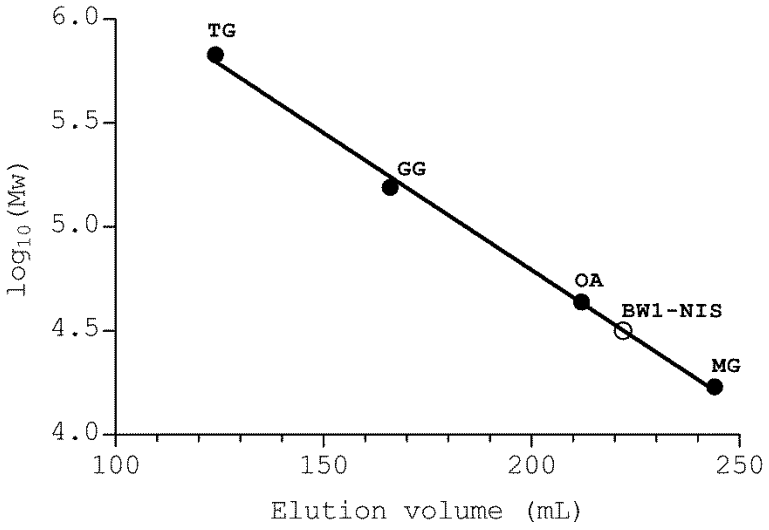

Fig. 5.7. Estimation of molecular weight of BW1-NIS. A molecular weight of 32 kDa is calculated for BW1-NIS from the calibration curve of the size exclusion chromatography column, and from a BW1-NIS elution volume of 222mL. Molecular weight standards thyroglobulin (TG), γ-globulin (GG), ovalbumin (OA), myoglobin (MG).

To assist in determining if BW1-NIS is haemocin, the gDNA of BW1 was tested for *hmcA* by real-time PCR. Both BW1 and RHH122, the other NIS-producing *H. haemolyticus* isolate, were negative for *hmcA*.

5.4 Discussion

The objective of this study was to understand the production, activity and chemical characteristics of BW1-NIS. We were able to show that enrichment was achieved with an ammonium sulfate saturation of 60% or more, and activity was only detectable when the absorbance (OD_{600}) of the culture broth was 1.5 or greater. BW1-NIS was resistant to inactivation by heat, pH extremes and DNAse I, but was degraded by proteinase K. Spectrum of activity testing showed that BW1-NIS was active against all tested NTHi, Hib, and some *H. haemolyticus*, but no bacteria from ten other clinically relevant genera. The molecular size of BW1-NIS was approximately 32 kDa, and BW1 gDNA was negative for the presence of *hmcA*.

We determined that ammonium sulfate concentrations of 60, 70 and 80% saturations were equally effective at enriching BW1-NIS (Fig. 5.1), and as result 70% saturation was used for all subsequent enrichment procedures. This working concentration differs from the 55% saturation we used for preparation of enriched cell-free culture broth in Chapter 4, a concentration which was chosen from the methods used for the discovery of haemocin (Venezia and Robertson, 1975). It is conceivable that re-screening our *H. haemolyticus* collection for NIS by enriching with 70% ammonium sulfate saturation may uncover other NIS producing isolates.

For the characterisation of BW1-NIS, it is informative to compare it with haemocin. As was the case for haemocin (Venezia and Robertson, 1975), the activity in the culture broth (presumably related to amount of BW1-NIS present) increased rapidly during the late growth phase, with the increase slowing but continuing during stationary phase (Fig. 5.2). However, BW1-NIS required seven hours of culture and a population of approximately 1.2×10^9 CFU/mL to be first detectable, whereas haemocin was detectable after only 4 hours of growth, and a population of approximately 5×10^7 CFU/mL. This discrepancy may prove to be revealing of a difference in the specific activity or mechanism for induction of the production of the two substances. There were some similarities in the spectrum of activity of the two substances, with BW1-NIS inhibiting 100% of *H. influenzae* and 57% of *H. haemolyticus* (Table 5.1), and haemocin inhibiting 93% of NTHi and 75% of *H. haemolyticus* (Venezia et al., 1977). However, when haemocin and BW1-NIS were tested on a common set of *H. haemolyticus* isolates (n = 30), although a regression of the clearing zone annular radii produced a significant positive relationship (Fig. 5.3), 12 isolates were not inhibited by either substance, 4 isolates were inhibited by BW1-NIS only, and 3 were inhibited by only haemocin. Also, there were other clear differences in the spectrum of activity of the two substances: *E. coli* was inhibited

by haemocin (Streker et al., 1981; Venezia and Robertson, 1975; Venezia et al., 1977), but not by BW1-NIS (Table 5.1); Hib are immune to the effects of haemocin (Murley et al., 1997, 1998) but were inhibited by BW1-NIS.

Using size exclusion chromatography, we showed that BW1-NIS had an estimated molecular size corresponding to a monomeric protein with a molecular weight of 32 kDa (Fig. 5.6, 5.7). Consistent with this estimate, BW1-NIS was retained by a 10 kDa MWCO centrifugal filter and 3.5 kDa dialysis tubing. However, we could not distinguish from our data whether this represents the molecular size of a BW1-NIS polypeptide, or a homo- or hetero-oligomer assembly thereof. In this regard, we note that the molecular size of haemocin was initially estimated, by retention in membrane filters, to be between 50 and 100 kDa (Venezia and Robertson, 1975); the purified polypeptide in the denatured state was less than 14 kDa by SDS-PAGE (Venezia et al., 1977); and the molecular size predicted for the mature processed polypeptide using the gene sequence is only 5.0 kDa (Murley et al., 1998). These differences between BW1-NIS and haemocin, together with our RTPCR results which showed that *hmcA* was absent from BW1, are consistent with the evidence first presented in chapter 4, which showed that BW1-NIS and haemocin are different substances.

Previously, in Chapter 4, we suggested that the anti-NTHi activity of BW1-NIS is consistent with either a siderophore, quorum sensing molecule or bacteriocin. The results in this Chapter provide some information relevant to answering this question. It appears unlikely that BW1-NIS is a siderophore, as these are usually low molecular weight molecules, of 0.5 kDa to 1.5 kDa (Hider and Kong, 2010), whereas BW1-NIS is approximately 30 kDa. The quorum sensing acyl homoserine lactone molecules, which are common to Gram-negative bacteria, appear as unlikely candidates, also due to their very small molecular size. The degradation of BW1-NIS activity by proteinase K suggests that it is a peptide, which is also not consistent with

siderophores or quorum sensing molecules. Bacteriocins are peptides, have narrow spectrum of activity, molecular weight between 1 and 70 kDA, and may be resistant to extremes of heat and pH (Yang et al., 2014), and as such are fully consistent with the known characteristics of BW1-NIS. Indeed, although differences have already between outlined above, BW1-NIS and haemocin, the bacteriocin produced by Hib, share many characteristics, including similar spectrum of activity, resistance to extremes of heat and pH, and susceptibility to proteases (Venezia and Robertson, 1975)..

In conclusion we have shown that BW1-NIS is most likely a peptide, possibly a bacteriocin, with very specific activity against the closely related species *H. influenzae* and some *H. haemolyticus*. We suggest that strains of *H. haemolyticus* that produce BW1-NIS may be useful as probiotics, and that further studies of the structure and function of BW1-NIS are required.

Chapter 6.

Purification and sequencing of the NIS produced by *H. haemolyticus* (Hh-NIS)

Figure 6.1 “SDS-PAGE analysis and NIS activity in SEC separated BW1-NIS”, from this chapter, has been incorporated into the publication “An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like substance that inhibits the growth of nontypeable *Haemophilus influenzae*” by Latham, R. D., Gell, D. A., Fairbairn, R. L., Lyons, A. B., Shukla, S. D., Cho, K. Y., . . . Tristram, S. G.’ which was published in the International Journal of Antimicrobial Agents in April 2017 (doi:10.1016/j.ijantimicag.2016.12.010).

6.1 Introduction

In chapter 4, we showed that *H. haemolyticus* isolates BW1 and RHH122 produced a diffusible substance that inhibits the growth of *H. influenzae* under the assay conditions employed. In chapter 5, we characterised some of the chemical and physical properties of an NTHi inhibiting substance (NIS) made by BW1, showing that it had narrow spectrum activity against *H. influenzae*, and determined that it was likely to be a proteinaceous substance, possibly a bacteriocin, with molecular size of approximately 32 kDa. We now sought to continue this investigation, by describing the protein and gene sequences of the NIS produced by BW1 (BW1-NIS), as well as the NIS produced by RHH122 (RHH122-NIS).

In this Chapter, we used size exclusion (SEC) and reverse-phase high-performance liquid chromatography (RP-HPLC) to obtain highly purified BW1-NIS and RHH122-NIS. Tryptic fragments of these preparations were analysed by mass spectrometry, and peptide sequences identified by matching the molecular mass of trypsin digest fragments against a hypothetical trypsin digest of *H. haemolyticus* proteins, derived from published genome sequence data. The candidate gene for the NIS made by BW1 and RHH122 (Hh-NIS) was cloned into in *E. coli*, expressed and tested for activity. The prevalence and variability of the ORF for the *Hh-NIS* gene were determined in Genbank, as well as in our bacterial collection. The structure and function of the protein were predicted with structure homology modelling.

6.2 Methods

6.2.1 Growth Media

For this chapter, media for growth of *H. haemolyticus* was filter sterilised tryptone soya broth (Oxoid, CM1065) supplemented with HTM supplement (Oxoid, SR0158) and Vitox (Oxoid, SR0090A) (sTSB).This media was used in preference to sBHI, as testing showed that it

supported growth of *H. haemolyticus* and production of NIS, as well as clearer resolution of on silver-stained protein bands on SDS-PAGE gels.

6.2.2 Enrichment of cell-free culture broths

Similar to Chapters 4 and 5, cell-free culture broth was enriched by ammonium sulfate precipitation (Chapter 2.7), but with the precipitate formed at 50% saturation discarded, and the subsequent precipitate formed at 70% saturation retained, and hereafter is referred to as the 50-70% fraction.

6.2.3 Increased-sensitivity agar well diffusion assay for NIS activity

For the investigation of Hh-NIS production by gene-positive isolates, bacteria were grown in sTSB, then the 50-70% fraction of the cell-free culture broth dissolved in a volume of DPBS equal to 1/100th (cf. 1/20th) of the culture broth volume.

6.2.4 Size exclusion chromatography

For separation of samples by size exclusion chromatography, a Superose 12 HR 10/300 GL column (GE Healthcare Life Sciences, 17-5173-01), with a bed volume of 24mL was used. The buffer was 0.15M Na phosphate pH 7.0, which ran at a flow rate of 0.75mL/min, at 21°C.

6.2.5 Reversed-Phase High-performance liquid chromatography

Samples were separated with a reversed phase high-performance chromatography (RP-HPLC) column (Waters, C4), with 0.1% trifluoracetic acid (TFA) for gradient buffer A, and acetonitrile with 0.1% TFA for gradient buffer B. Fractions were collected manually.

6.2.6 Mass spectrometry analysis and database search

This was performed by R. Wilson. SDS-PAGE gel slices, which contained protein bands, , were prepared for mass spectrometry analysis by rinsing twice in 0.5L of water, incubation for 10 minutes in a solution containing 50% acetonitrile and 0.05M ammonium bicarbonate, then for 5 minutes in 100% acetonitrile, and finally drying by evaporation. An in-gel tryptic digest

of the bands was performed as described previously (Wilson et al., 2008). Briefly, gel slices were rehydrated for 1 hr at 4°C in 20µL of digest buffer (40mM ammonium bicarbonate and 10% acetonitrile) containing 20ng/µL proteomics-grade trypsin (Sigma). A further 20µL of digest buffer was added to the samples, before incubation overnight at 37°C. The supernatants were then removed, incubated for 1 hour at 4°C with 30µL of peptide extraction buffer (50% acetonitrile in 0.1% formic acid), sonicated for 10 minutes in a water bath, and then dried. The dried tryptic peptides were then reconstituted with 20µL of HPLC Buffer A (5% acetonitrile in 0.2% formic acid) before analysis by nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS), using an LTQ-Orbitrap XL (ThermoFisher Scientistic). For nano LC-MS/MS, the tryptic peptides were first loaded at 0.05mL/min onto a C_{18} capillary trapping column (Peptide CapTrap, Michrom BioResources), which was controlled by a Separations Module (Waters, Alliance 2690). Peptides were then separated on an analytical C_{18} nano-column (New Objective, PicoFrit, 10cm with 15µm i.d pulled tip), controlled by a pump (Thermofisher Scientific, Surveyor MS Pump Plus) running at a flow-rate of 200 nL/min, over a 4-step gradient of 100% Buffer A to 100% Buffer B (90% acetonitrile in 0.2% formic acid). The gradient steps were as follows: 0 – 10% B, 7.5 min; 10 – 25% B, 50 min; 25 – 55% B, 20 min; 55 – 100% B, 5 min; then hold at 100% B, 15 min. The software used for control of the LTQ-Orbitrap XL was Xcalibur 2.0 (ThermoFisher Scientific), which was operated in data-dependent acquisition mode, with survey scans acquired in the Orbitrap at a resolving power of 60,000. The MS/MS spectra, for the eight most intense ions from the FT survey scan, were concurrently acquired in the LTQ mass analyser. Charge state filtering was used for unassigned and singly-charged precursor ions which were not selected for fragmentation, as well as dynamic exclusion (repeat count 1, repeat duration 1 sec, exclusion list size 500). The fragmentation conditions used were: 35% normalized collision energy, 0.25 activation q, 30ms activation time, and a minimum ion selection intensity of 500 counts. For database searching,

the MS/MS .raw files generated by Xcalibur 2.0 were searched against the *Haemophilus haemolyticus* database, downloaded from NCBI on 30 September 2016. Semi-tryptic searches, using parent ion and fragment ion mass tolerances of 10 ppm and 0.5 Da, were performed using X!Tandem running in CPAS (Rauch et al., 2006). The probabilities of the protein and peptide matches were calculated (Nesvizhskii et al., 2003) using the Protein Prophet and Peptide Prophet algorithms.

6.2.7 Cloning and expression

A PCR product was made using BW1 gDNA as template, with primers QVVGN.F (5'-ATTACATATGCAGGTAGTGGGAAATGTATCA-3') and FGGTKN.R (5'-TTATCTCGAGTTAATTTTTAGTACCGCCAAA-3'), which coded for the mature peptide without the signal peptide (Fig. 6.5). A truncated version was also made, using primers DSSIPN.F (5'- ATTACATATGGACAGTAGTATTCCTAATGAT-3') and FGGTKN.R. The PCR amplicons were separately annealed into plasmid PET28A, which had been cut previously with restriction enzymes NdeI and XhoI, using T4 DNA ligase. *E. coli* DH5 alpha was transformed with this construct and clones were verified by Sanger sequencing. *E. coli* expression strain BL21 was transformed with the plasmids and a starter culture was grown overnight in LB with 24 μg/mL kanamycin antibiotic; the overnight culture was inoculated into a fresh culture (1 L LB with kanamycin), induced with 1 mM IPTG at an absorbance (OD_{600}) of ~0.6, and grown for 4 hours at 37 °C. Cells were collected by centrifugation at 4,000 x g for 10 minutes, and resuspended in buffer (0.3M NaCl, 0.025M sodium phosphate, 10 mM imidazole, pH 7.1) containing DTT (1 mM) and PMSF (0.2 mM), and lysed by sonication (Sonics 500-Watt probe sonicator). The insoluble fraction was removed by centrifugation (17,000 rpm, Sorvall SS34 rotor, 4 °C). The expressed proteins were then purified by affinity chromatography with Nickel HTC agarose resin (Gold Biotechnology, R-202).

6.2.8 RTPCR and Sanger sequencing

An alignment of genes from Genbank (Appendix 3) were used to design primers for RTPCR and Sanger sequencing. Primers OMPA-726F (5'-TGTAAGGTGTGAAATCCATTTATCG-3') and OMPA-851R (5'-GGCGTTGAGATATATGACAGTAG-3'), which annealed within the ORF, were used for SYBR Green RTPCR testing, and primers OMPA-spanF (5'-AATCCAGTATTAGTTGTTGATGC-3") and OMPA-spanR (5'-CTTGGTTGTTTATTGTTAATGTAG-3'), annealing outside the ORF, were used for PCR amplification and Sanger sequencing.

6.3 Results

Size exclusion chromatography fractions of BW1-NIS and RHH122-NIS preparations were assessed for activity by agar well diffusion assay, and visualised by SDS PAGE. A band of ca. 30 kDa was present in column fractions that retained NIS activity, from BW1 and RHH122, and was not present in the equivalent fractions from two non-producing strains, BW39 and BWOCT3 (Figure 6.1, SDS-PAGE analysis of SEC fractions, for producing strain BW1 and non-producing strain BW39 only).

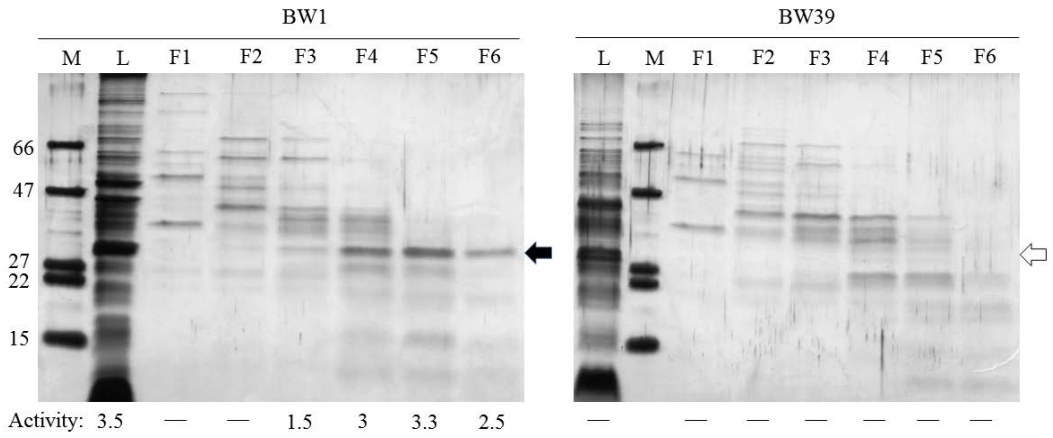

Fig. 6.1. SDS-PAGE analysis and NIS activity in SEC separated BW1-NIS. Load fraction (L) and elution fractions (F1–F6) from SEC were separated on 10% Tris-tricine SDS-PAGE and stained with silver. Molecular weight size standards (M) are indicated, together with activity measured as clearing zone annular radius (mm). A 30-kDa polypeptide was evident in BW1 samples (solid arrow head), but not in fractions from non-NIS producing *H. haemolyticus* isolate BW39 (open arrow head).

The RHH122 SEC fraction with the highest abundance of the 30-kDa band (not shown, comparable to Fig. 6.1, BW1, F5) was separated by RP-HPLC (Fig. 6.2). Then, with the acetonitrile removed by evaporation and lyophilisation, the RP-HPLC fractions were resuspended in DPBS, the NIS activity was measured by agar well diffusion assay, and molecular weight of separated products determined by SDS-PAGE. Only the fraction collected at 20.3-21.2 minutes had NIS activity (Fig. 6.3), and a ca. 30 kDa band (Fig. 6.4), which was excised and subject to in-gel trypsin digest, and the derived peptides analysed by mass spectrometry.

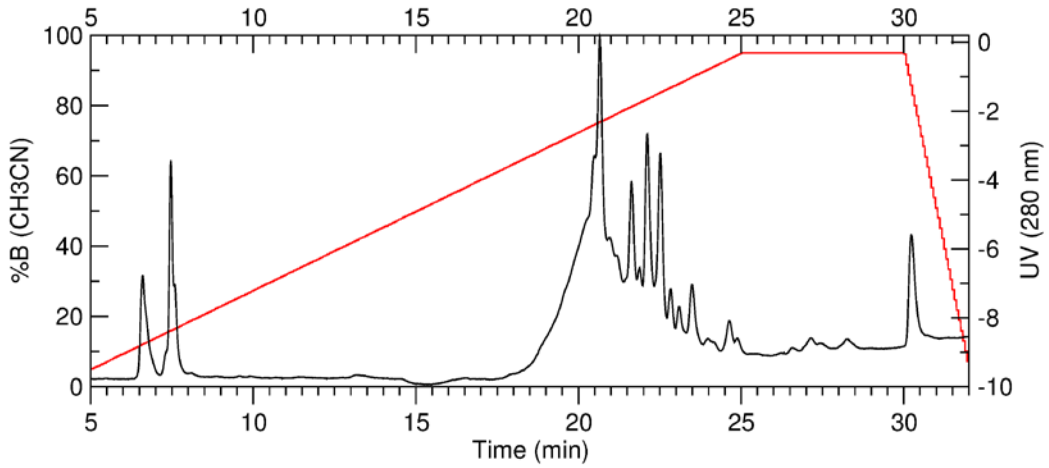

Fig 6.2. RP-HPLC separation of SEC purified RHH122-NIS. The peak at 20.6 minutes has NIS activity (Fig. 6.3) and a 30 kDa band on SDS-PAGE (Fig. 6.4). Red line is the gradient, black line is the UV.

The resulting masses from mass spectrometry analysis were compared against hypothetical tryptic digests, of the predicted proteins in Genbank, derived from the whole genome sequencing bioprojects of 13 *H. haemolyticus* strains (M19107, M19501, M21127, M21621, M21639, HK386, CCUG 24149, CCUG 39154, CCUG 12834, 27P25, 1P26, 3P5, 11P18). The comparison revealed a match for a hypothetical protein (EGT80255), described as a putative OMPA-like protein, identified from an ORF (*Hh-NIS*, AFQN01000044.1) in *H. haemolyticus* strain M19107 (AFQN). This strain was a carriage isolate, not associated with disease, from a study of invasive *H. haemolyticus* (Jordan et al., 2011). The hypothetical protein included a signal peptide (Fig. 6.5, sequence in blue), predicted by SIGNALP 4.1 (Petersen et al., 2011), that would be cleaved off between amino acid residue 22 and 23, AQA-QV. The position of this signal peptide cleavage site was confirmed with the presence, in the mass spectrometry analysis, of the peptide QVVGNVSTDTNQTR (Fig 5.5. first tract of sequence in red, after signal peptide). With the same methods of purification and mass spectrometry analysis applied

to BW1-NIS, an identical result was obtained, and BW1-NIS and RHH-122 NIS were then referred to as “Hh-NIS”.

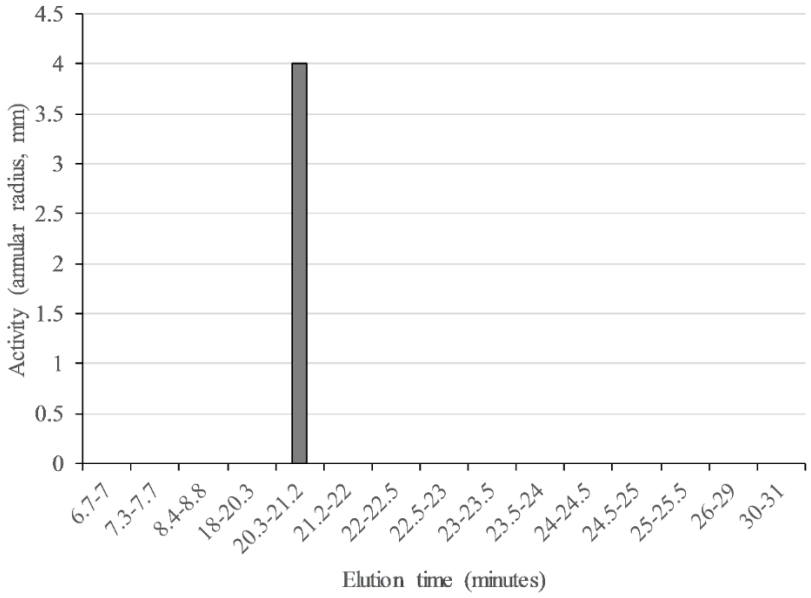

Fig. 6.3. Activity in RP-HPLC fractions from SEC purified RHH122-NIS. Activity from agar well diffusion activity, with NTHi strain NCTC 4560 as indicator.

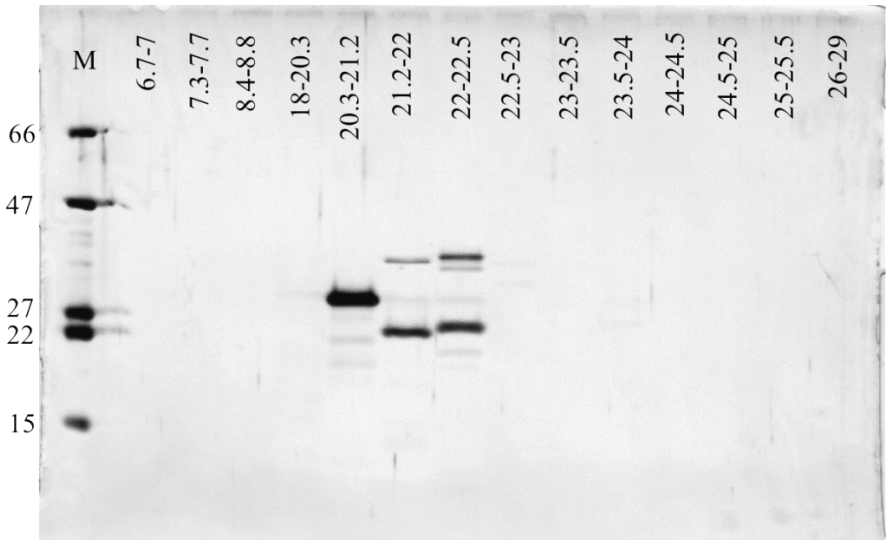

Fig 6.4. SDS-PAGE analysis of RP-HPLC purified RHH122-NIS. Lane M, molecular weight marker (kDa). Other lanes, RP-HPLC fractions, elution times (minutes) as shown.

MSVINKTILAVVVCGVVSVAQAQVVGNVSTDTNQTRYIKIKAGEKDGKAGVEIYDSSIPNDP
AVLSKTANNKGQSFEKMAERADKWISHLTGVAKKDKNGVIVAKMNKMPNLTLIMPDHRGL
GRLSFKQVGNQDTYFGEWENVDAATSAAKNVSVYYAGSDPTKTLPSGKATYTVEGINKYG
NFNSRLMKGTFDVDFERASISGYLSKPNLSLSIESKIDKTNATFEGIAKAEGVIGKSEGRFYGA
KAEGLAGMATFASKPEYNTAFGGTKN

Fig. 6.5. RHH122-NIS is EGT80255 from *H. haemolyticus* M19107. Blue is signal peptide. Red are hypothetical tryptic fragments with masses matching the RHH122-NIS tryptic fragment masses. Standard single letter amino acid code.

For the ultimate verification that Hh-NIS was the active peptide, the *Hh-NIS* ORF, minus the signal peptide was cloned from BW1 into *E. coli*, expressed (Fig. 6.6, lane 5) and purified on Ni-affinity resin (Fig. 6.6, lane 7), followed by size exclusion chromatography (Fig. 6.7), then tested for activity. The resulting preparation had the same NIS activity as BW1-NIS and RHH122-NIS preparations (Fig. 6.8, 3-1), confirming that the mass spectrometry analysis had identified the correct protein. A recombinant truncated Hh-NIS, in which 32 residues were removed from the N-terminus, showed similar level of expression in *E. coli* (Fig. 6.6. lanes 4, 6) and mobility on SEC (not shown), suggesting it was substantially folded, yet was inactive. (Fig 6.8, 2-1). This sample provides a control, showing that it is unlikely that the NIS activity arose from a contaminating *E. coli* protein.

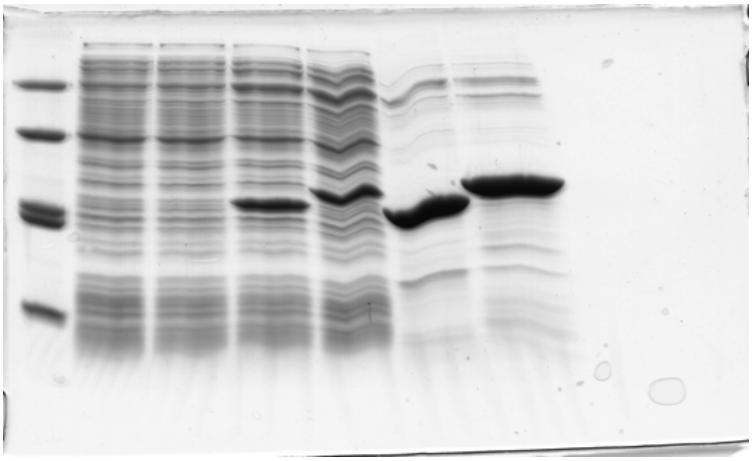

Fig. 6.6. SDS-PAGE analysis of recombinant Hh-NIS expression and Ni-affinity purification. Lane 1, molecular weight markers, same as Fig. 6.4; lane 2, clone 2-1 pre-induction lysate; lane 3, clone 3-1 pre-induction lysate; lane 4, lysate from clone 2-1 induced with 1 mM IPTG for 3 hours at 37°C; lane 5, lysate from clone 3-1 induced with 1 mM IPTG for 3 hours at 37°C; lane 6, clone 2-1 eluted from Ni-affinity; lane 7, clone 3-1 eluted from Ni-affinity.

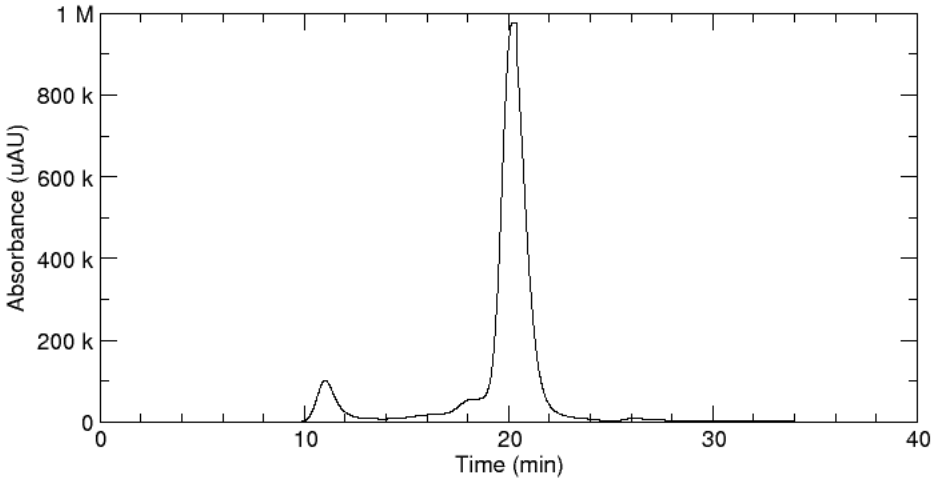

Fig. 6.7. SEC purification of recombinant Hh-NIS. Ni-affinity purified Hh-NIS from clone 3-1 was passed over a SEC column, and the 19 to 21.5 minute eluate was collected for activity testing (Fig. 6.8).

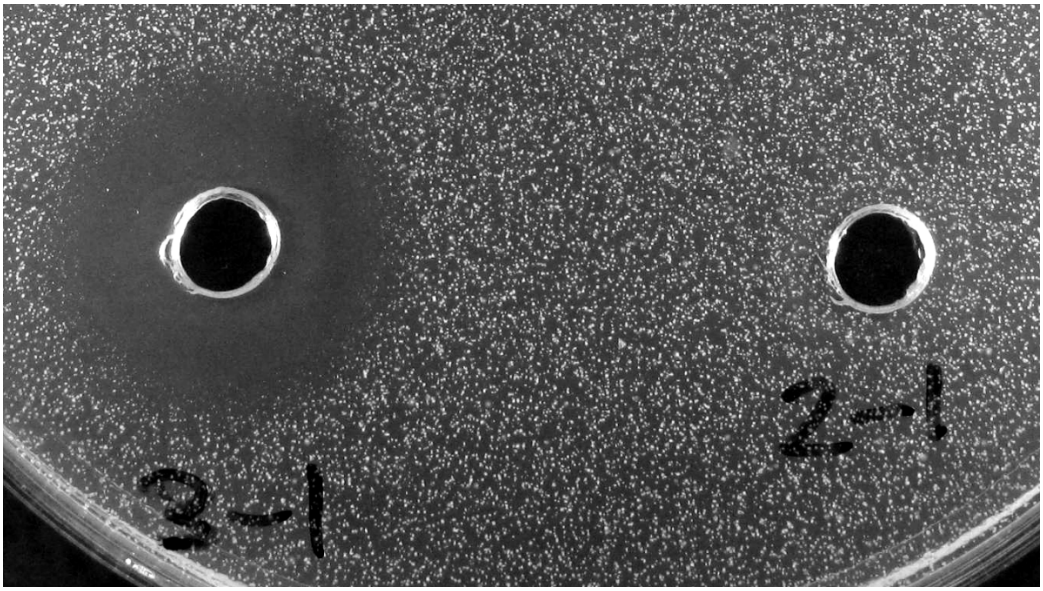

Fig. 6.8. Activity of recombinant Hh-NIS. There was NIS activity in recombinant Hh-NIS made in *E. coli* with the *Hh-NIS* ORF minus the signal peptide (3-1). In a truncated version, with 32 residues missing from the N-terminus, there was no activity (2-1). Agar well diffusion assay with NTHi reference strain NCTC 4560 as indicator.

Genes similar to the *Hh-NIS* ORF (AFQN01000044.1) were identified in whole genome sequencing projects in Genbank for *H. haemolyticus*, *H. influenzae*, and *H. quentini* using NCBI blastn. Out of the 13 *H. haemolyticus* bioprojects, including M19107, 6 strains (46%) had ORF sequences that were 75% to 100% similar to *Hh-NIS* (Table 6.1). This set of 6 strains included 2 isolates (M21639, M21227) which had been identified as invasive (Jordan et al., 2011). Of the 126 *H. influenzae* bioprojects, 3 (2%) had genes that were 75% similar to the *Hh-NIS* ORF. In the 3 *H. quentini* bioprojects, all 3 (100%) had ORF sequences that were 97% similar to the *Hh-NIS* ORF.

Table 6.1. Genbank whole genome sequencing projects with genes similar to the *Hh-NIS* ORF. Comparisons between *Haemophilus* spp. in Genbank, including 13 *H. haemolyticus*, 126 *H. influenzae* and 3 *H. quentini*, and the 816 bp *Hh-NIS* ORF (AFQN01000044.1).

Species-strain (Genbank accession code)	**Similarity (%)**
Hh-M19107 (AFQN)	816/816 (100)
Hh-11P18 (LCTK)	812/816 (99)
Hq-C860 (MDJC)	795/816 (97)
Hq-K068 (MDJB)	795/816 (97)
Hq-MP1 (MCII)	795/816 (97)
Hh-M21639 (AFQR)	795/816 (97)
Hh-27P25 (LCTH)	605/805 (75)
Hh-M21127 (AFQP)	605/805 (75)
Hh-CCUG 24149 (LZDL)	600/804 (75)
Hi-1104 (JFZM)	438/582 (75)
Hi-584 (JFZL)	438/582 (75)
Hi-411 (JFZK)	438/582 (75)

In our collection of 100 *H. haemolyticus* isolates we examined, the prevalence and variability of the ORF for *Hh1-NIS*, and the NIS activity in the gene-positive isolates. Using primers annealing within the ORF, 15 (15%) of the isolates were positive for *Hh-NIS* by RTPCR, and using primers annealing outside of the ORF, 11 of these 15 (11% of total) could be amplified by PCR and then sequenced (Table 6.2). The other four that were positive in the RTPCR (BW36, L117, L152, L153) did not amplify in the second PCR (and so were not sequenced). The sequences from six isolates (BW1, RHH122, BW15, BW18, NF4, NF5) were identical to

the *Hh-NIS* ORF, and the other five (BW5, CF14, L19, NF6, NF11) were divergent (Table 6.2). Of the 156 NTHi isolates in our collection, none (0%) were positive by RTPCR for the presence of the *Hh-NIS* ORF.

Expression of Hh-NIS by the RTPCR-positive isolates was assessed using an increased-sensitivity agar well diffusive assay. The NIS activity was measured in 100-fold (cf. 20-fold) enriched solutions, and with solution concentrations equalised for the culture density (Table 6.2). The isolates with ORF sequences identical to *Hh-NIS,* all showed evidence for Hh-NIS production, however production was higher in BW1 and RHH122 than any of the other isolates (Table 6.2). One isolate (NF6), with an ORF sequence divergent from M19107, showed signs of NIS activity, although at a lower level than the isolates with sequences identical to *Hh-NIS.* There was NIS activity produced by the isolates BW36, L152 and L153, which could not be sequenced, even though they were RTPCR-positive.

Table 6.2. *Hh-NIS* ORF prevalence and variability, and NIS activity, in our *H. haemolyticus* collection. The 100 *H. haemolyticus* isolates in our collection were tested for prevalence and variability of the *Hh-NIS* ORF (accession AFQN01000044.1) by RTPCR and sequencing, and for evidence of production of Hh-NIS by an increased-sensitivity agar well diffusion assay. Isolates for which no sequence similarity is available (NA), that did not amplify with the sequencing PCR, using primers annealing outside the ORF. NA, not available. * includes gaps. ND, not determined. Culture density, absorbance (OD_{600nm}). Activity, annular radius (mm).

Isolate	RTPCR	Sequencing PCR	Sequence similarity (%)	Activity	Activity, equalised for culture density
BW1	+	+	816/816 (100)	5.5	3.75
BW5	+	+	804/816 (99)	0	0
BW15	+	+	816/816 (100)	2	0
BW18	+	+	816/816 (100)	3	0
BW36	+	-	NA	3	ND
CF14	+	+	783/816 (96)	0	0
L19	+	+	706/830* (85)	0	0
L117	+	-	NA	0	0
L152	+	-	NA	3	0
L153	+	-	NA	3	0
NF4	+	+	816/816 (100)	2	0
NF5	+	+	816/816 (100)	3.5	2
NF6	+	+	783/816 (96)	1	0
NF11	+	+	785/816 (96)	0	0
RHH122	+	+	816/816 (100)	5.5	3.5

Using SWISS-MODEL for protein structure homology modelling (Biasini et al., 2014), HpuA from *Kingella denitrificans* (protein data bank accession 5ee4; crystallized in complex with human oxy-haemoglobin) was identified as the most similar template, and a model for Hh-NIS protein was generated (Fig. 6.9). The software indicated the presence of a beta barrel in the C-terminal region of Hh-NIS with high confidence (Fig. 6.9, blue). Prediction for the N-terminal region was of low confidence (Fig. 6.9, red); and is the region corresponding to the part of HpuA that binds to haemoglobin (Wong et al., 2015).

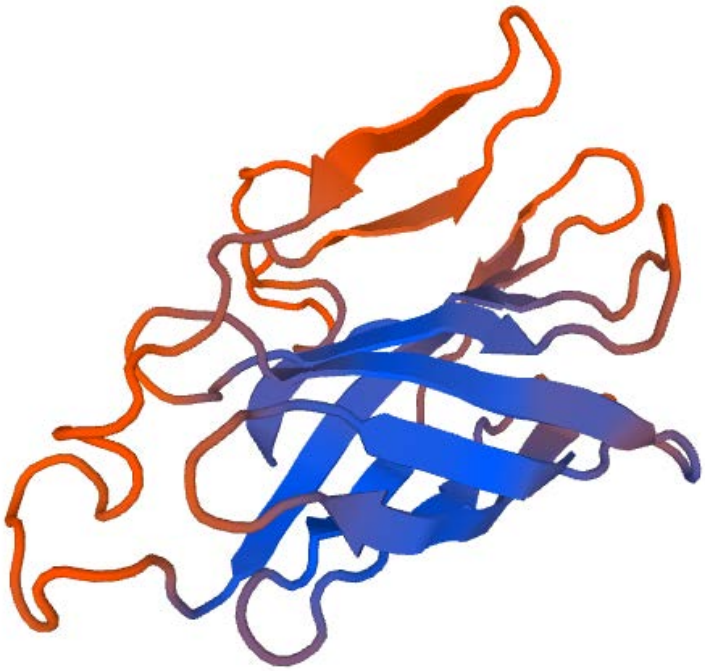

Fig. 6.9. Model structure of Hh-NIS. Generated by SWISS-MODEL, based on homology to HpuA from *Kingella denitrificans*, with cartoon view shown. Blue, high confidence; red, low confidence.

Protein structural homology modelling was also conducted on the translations of the *Hh-NIS* ORF, identified by the blastn algorithm in Genbank whole genome sequencing projects (Table 6.1), as well as from the isolates in our collection (Table 6.2). As was the case for Hh-NIS in BW1, RHH122, and M19107, all structures were most similar to HpuA from *Kingella denitrificans*, and the structure of the beta-barrel (Fig. 6.9, blue) was consistently predicted, but there was variability in the N-terminal regions.

6.4 Discussion

In this study, the NTHi inhibiting substances (NIS) produced by BW1 and RHH122 were purified, and then with mass spectrometry analysis, shown to be EGT80255 (Hh-NIS), a hypothetical protein from an isolate of *H. haemolyticus* (M19107). Finally, a structure of Hh-NIS was constructed, based on its homology with HpuA.

The mature Hh-NIS peptide was 27 kDa (subsequent to cleavage of the signal peptide), similar but not identical to the 32 kDa predicted in Chapter 6, and the approximate 30 kDa observed on SDS-PAGE (Fig. 6.1, 6.4, 6.6). Although this small discrepancy may be within the normal limits of error expected for SEC, because elution time scales with the logarithm of the molecular weight, this consistent over-estimation of the molecular size, could also be a “gel shift” (Rath et al., 2009), which occurs in the presence of SDS, and causes protein shape as well as molecular size to contribute to electrophoretic mobility.

The structural gene for Hh-NIS *(Hh-NIS*, accession AFQN01000044.1) was identified from a carriage isolate (M19107) in a study of the genomes of *H. haemolyticus* isolates causing disease (Jordan et al., 2011). Although the M19107 strain was not associated with pathogenicity, two invasive isolates (M21639, M21227) from the same study carried the gene, albeit with 75% similarity. Although noteworthy, the small number of isolates in that study - two carriage

isolates and three associated with disease - do not allow us to make any meaningful conclusions about the relationship between pathogenicity and presence of *Hh-NIS*. Indeed, the rarity of *H. haemolyticus* disease in humans will make challenging for any *in vivo* study of the association between Hh-NIS and pathogenicity.

In an examination of our *H. haemolyticus* collection, six isolates (BW1, RHH122, BW15, BW18, NF4, NF5) had sequences identical to the *Hh-NIS* ORF, but their NIS activity was highly variable, with BW1 and RHH122 consistently higher producers. Even with correction for culture density (Table 6.2), these differences were apparent, and therefore appear to be due to different levels of expression. To gain an understanding of the control of expression of Hh-NIS, an examination of the sequences in these six isolates upstream of the *Hh-NIS* ORF is suggested, as it is the likely position for a promotor.

The above suggested differences in sequences upstream of the ORF, could also explain the results for the *H. haemolyticus* isolates BW36, L152 and L153. These three isolates were positive for NIS activity and presence of *Hh-NIS* ORF, by RTPCR of an internal fragment, but were not sequenced due to lack of amplification of the sequencing PCR. The primers for the sequencing PCR had been designed to anneal adjacent to the ORF, and it is conceivable that the ORF in these isolates was 100% similar to that of M19107, but variability in one or both of the primer binding sites adjacent to the ORF had caused the PCR failure To determine if this is the case, the ORF of these three isolates should be sequenced, initially with primers that anneal within the ORF.

The prevalence of *Hh-NIS* in *H. quentini*, a rare cause of genitourinary tract infection (Horie et al., 2017) and neonatal bacteraemia (Hubbard et al., 2016), is higher than in other *Haemophilus* species. All three (100%) of the *H. quentini* Genbank genomes contained *Hh-NIS*, whereas in

H. haemolyticus, the rate of prevalence was 46% in the published genomes, and 15% in our collection by RTPCR. In *H. influenzae*, only 2% of published, and 0% in our collection were carrying *Hh-NIS*. Although the number of *H. quentini* strains examined was small, they may reflect the larger population, as this species is genetically relatively homogenous (Bruant et al., 2003; Quentin et al., 1990, 1993). However, we do not expect this high prevalence of *Hh-NIS* in *H. quentini* to be significant for the overall aim of our project, the assessment of the potential of *H. haemolyticus* to inhibit the growth of NTHi, as *H. quentini* is a resident of the genito-urinary tract (Glover et al., 2011), and *H. haemolyticus* and *H. influenzae* are resident of the respiratory tract, and so are unlikely to be in a competitive situation.

Although the mass spectrometry analysis described Hh-NIS as a putative OMPA-like protein, an experimentally verified function was provided by structural homology modelling. Using SWISS-PROT, amino acid sequences were compared against proteins with known function and experimentally determined tertiary structure, and the Hh-NIS protein was identified as most similar to HpuA from *Kingella denitrificans* and *Neisseria gonohorreae*. HpuA is an outer membrane protein reported to be required for the removal of haemoglobin from haemoglobin-haptoglobin complexes, and as such would appear to be of interest to us, as the haem requirement of *H. influenzae* and *H. haemolyticus* can be provided by haemoglobin. However, this apparent functional similarity is likely misleading, as HpuA binds only the globin protein of the haemoglobin complex (Wong et al., 2015), and the predicted structure of the ligand-binding region (Fig. 6.9, red colour) is of low confidence.

In this Chapter we determined the sequence of the Hh-NIS protein and the *Hh-NIS* ORF, and showed that recombinant Hh-NIS was active. Although some structural similarities to HpuA were shown, the mechanism for the inhibitory activity of Hh-NIS is not yet unknown, and will be the subject of the final experimental chapter of this thesis.

Chapter 7.

Mechanism of action of Hh-NIS

7.1 Introduction

In Chapter 6, BW1-NIS and RHH122-NIS were demonstrated to be a protein (Hh-NIS, accession EGT80255) in Genbank, deduced from a whole genome sequencing project (AFQN) of *H. haemolyticus* carriage isolate M19107. Recombinant *E. coli*, with the ORF (*Hh-NIS*) cloned in, expressed recombinant Hh-NIS. *In silico* structural modelling of Hh-NIS revealed similarity to HpuA, a haemoglobin binding protein present in some haem-auxotrophic pathogenic bacteria, including *Neisseria meningitidis*, *N. gonohorreae* and *Kingella denitrificans*. We now seek to understand the mechanism by which Hh-NIS inhibits NTHi growth.

In this Chapter, working with purified recombinant Hh-NIS, the absorbance spectrum was assessed, and highly purified fractions prepared. The NIS activity and minimum inhibitory concentration of the fractions were determined, and the effect of haemin supplementation on the activity of BW1-NIS and recombinant Hh-NIS were investigated.

7.2 Methods

7.2.1 Highly purified Hh-NIS

Recombinant Hh-NIS was purified by D. Gell. Briefly, Hh-NIS was expressed in *E. coli* and purified by Ni affinity chromatography, as described previously (Chapter 6.2.7). The 6-His tag was then removed by protease cleavage at an engineered thrombin site, and the tag was removed on Ni-affinity. The Hh-NIS fraction was then separated by high-performance cation exchange (UnoS1, Biorad). It was noted that two distinct fractions containing Hh-NIS (F16 and F17) eluted from the cation exchange column (Fig. 7.1). Wavelength scans (Fig. 7.2) revealed that, although F16 and F17 had similar absorbance at 280 nm, indicating similar protein concentrations (supported by SDS-PAGE, Fig. 7.3), F16 had strong absorbance at 414 nm, 541 nm and 576 nm, suggesting that it contained a bound cofactor. The cofactor occupancy

in F16 was assumed to be 100%, hence this fraction was referred to as the holoprotein. A second cation exchange step was performed on F17, and was successful in removing most of the remaining contaminating holoprotein, thus yielding an apoprotein fraction. The cofactor occupancy of the apoprotein fraction was estimated, based on a comparison of the ratio of absorbances at 415 nm and 280 nm. The A415:A280 for the apoprotein fraction was 0.19, and 2.5 for the holoprotein fraction, and as 0.19/2.5 is ~ 0.08, the cofactor occupancy in the apoprotein fraction was estimated to be 8%.

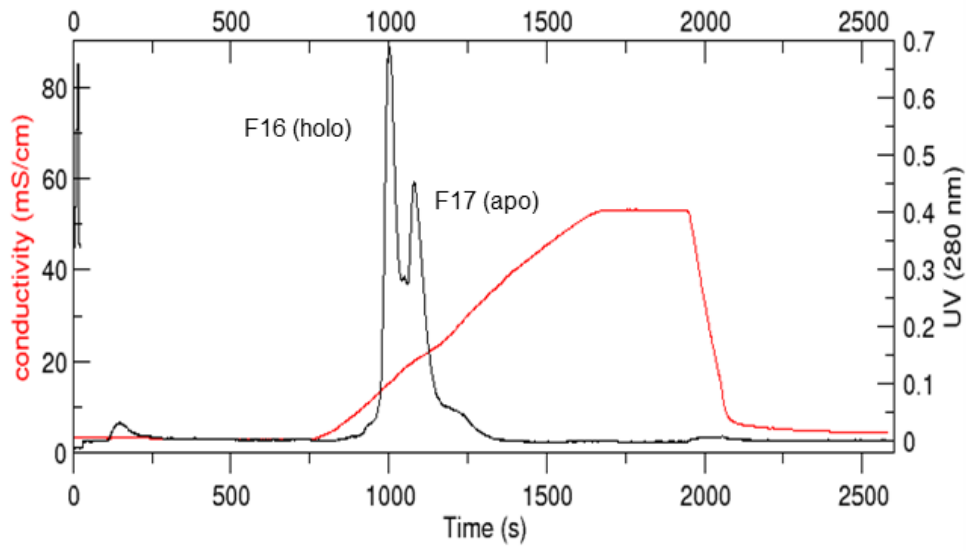

Fig. 7.1. Cation exchange chromatographic separation of recombinant Hh-NIS. Purified recombinant Hh-NIS was separated on a UnoS1 column, and two distinct fractions, F16 (holo) and F17 (apo) were collected. Data provided by D. Gell.

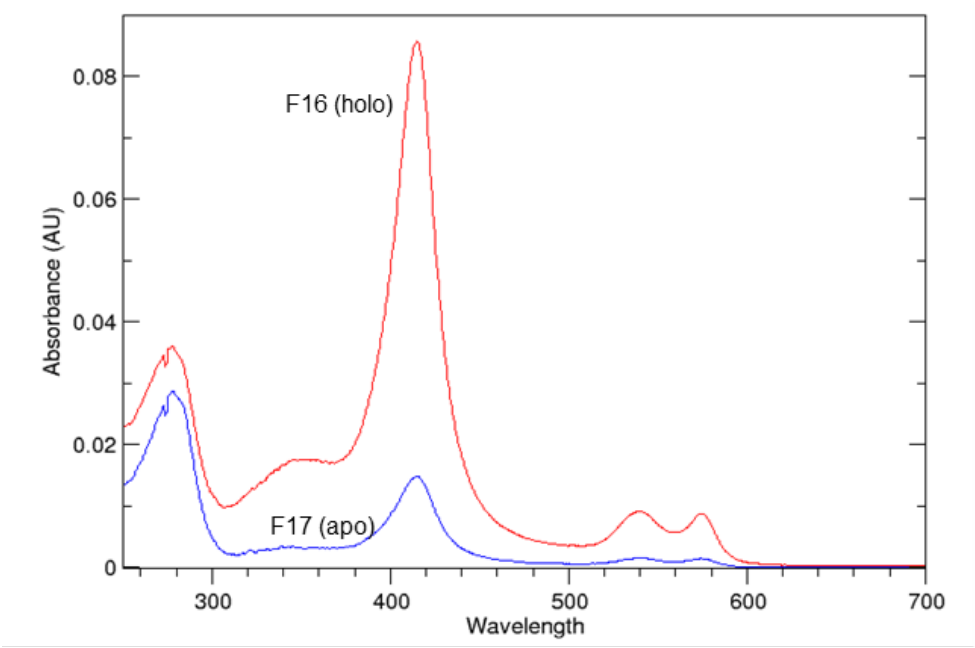

Fig. 7.2. Wavelength scan of Hh-NIS fractions eluted from cation exchange chromatography. When normalized to the 280 nm protein absorbance peak, the 414 nm, 541nm and 576 nm absorbance peaks were more abundant in F16 (holo, red line) than F17 (apo, blue line) fraction. Data provided by D. Gell.

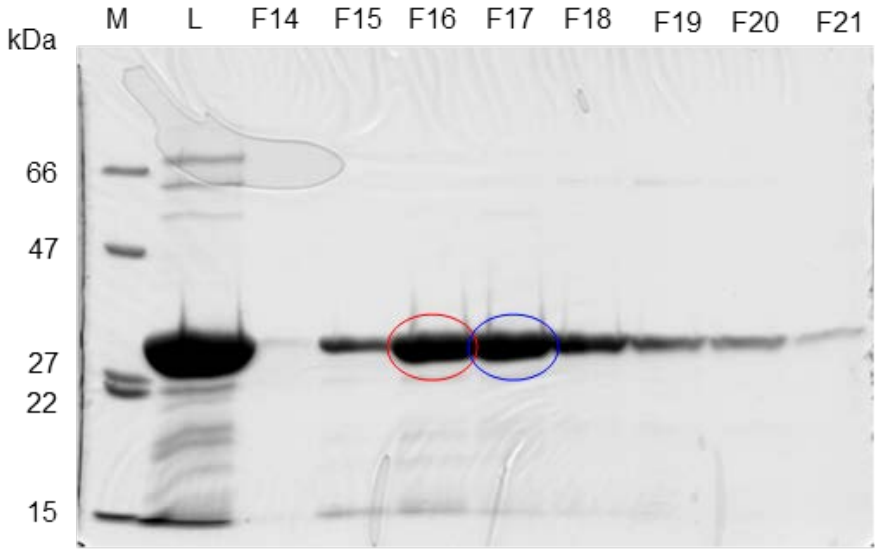

Fig. 7.3. SDS-PAGE of cation exchange fractions of recombinant Hh-NIS. Fractions 16 (F16, red oval) and 17 (F17, blue oval; see Fig. 2) contained predominantly the 27 kDa Hh-NIS protein. Protein size standards (M) and load material, from Ni-affinity step, (L) are also shown. Coomassie blue staining. Data provided by D. Gell.

7.2.2 Minimum inhibitory concentration of recombinant Hh-NIS

The minimum inhibitory concentration (MIC) of apoprotein and holoprotein preparations of Hh-NIS preparations was tested against NTHi. The dilutions were in 10-fold steps, and ranged from 0.00017µM to 1.7µM (holoprotein) and 0.0001µM to 1.0µM (apoprotein). Set up in 96-well tissue culture plates, each well contained 100µL sTSB and 100µL of the Hh-NIS preparation diluted in DPBS. Wells were inoculated to an absorbance of 0.05 (OD_{600}) with 5µL of NTHi strain NCTC 4560 or NCTC 11315, which had been grown for 9 hours on CA, then suspended in DPBS. Growth was assessed by measurement of absorbance (OD_{600}) with a plate reader, after incubation for 5.5 hours at 35°C with 5% CO_2. BW1 was included in the testing as a negative control

7.2.3 Haemin solution

A stock solution of 2000 µg/mL haemin (Sigma, 51280) was prepared in 0.1M NaOH, and stored in single-use aliquots at -20°C.

7.2.4 Haemin requirements for growth

The impact of haemin concentration on growth of BW1 and NTHi strain NCTC 4560 was assessed using broth culture in 96-well tissue culture plates. The haemin concentrations tested were from 40 µg/mL to 0.16 µg/mL, in two-fold steps. The media in each well was 200µL of TSB supplemented with Vitox (Oxoid, SR0090), NAD at 10µg/mL, and 4µL of haemin diluted in 0.1M NaOH. Each well was inoculated to an absorbance of approximately 0.05 (OD_{600}), with 5µL of either BW1 or NCTC 4560 suspension in DPBS, prepared from a 14-hour culture on chocolate agar. Growth was assessed by measurement of absorbance (OD_{600}) with a plate reader.

7.2.5 Effect of haemin on BW1-NIS activity in solid culture

Haemin was serially diluted in 0.1M NaOH, then 5µL combined with 20µL of a BW1-NIS preparation (Chapter 6.2.1, 6.2.2), with the resulting mixtures containing 40 µg/mL to 0.16 µg/mL haemin, in 2-fold steps. The NIS activity of the mixtures were tested in agar well diffusion assays, with NTHi strains NCTC 4560 and NCTC 11315 as indicators.

7.2.6 Effect of recombinant Hh-NIS in haemin-limited broth culture

The effect of recombinant Hh-NIS on growth of NTHi strain NCTC 4560 and BW1 was assessed in haemin-limited broth culture in 96-well plates. Haemin concentrations used were 0.625 µg/mL and 5 µg/mL, with 7.7µM of recombinant Hh-NIS holoprotein, which was equimolar to 5 µg/mL haemin. Each well contained 200µL of TSB supplemented with Vitox. NAD at 10µg/mL, 4µL of haemin diluted in 0.1M NaOH, and 5µL of the holoprotein preparation. Each well was inoculated to an absorbance of approximately 0.05 (OD_{600}), with 5µL of a DPBS suspension of either BW1 or NCTC 4560, prepared from a 14-hour culture on chocolate agar. Growth was assessed by measurement of absorbance (OD_{600}) with a plate reader.

7.3 Results

The recombinant Hh-NIS preparation was red-brown coloured, which led us to determine the visible absorbance spectrum. There was a strong absorbance peak at ~414 nm, which was reminiscent of the Soret band seen in metalloporphyrins and haemoproteins. To investigate this further, the absorbance spectrum of Hh-NIS was compared with spectra of the haemoprotein, haemoglobin (Hb). The wavelength scan of purified recombinant Hh-NIS identified a strong absorption peak at 414 nm, and two lesser peaks at 541 nm and 576 nm, and was highly similar to the spectrum of oxy-haemoglobin (Fig. 7.4), which contains haem with an Fe(II) metal, and an O_2 ligand. Met-haemoglobin, in which the haem is in the Fe(III) oxidation state, had only a

405 nm peak in this region. As such, Hh-NIS was thought to contain haem in which the iron was in the Fe (II) state and potentially also had an O_2 ligand bound. This result, which showed that Hh-NIS binds haem, led us to speculate that haem has a role in the activity of Hh-NIS, which was intriguing, as *H. haemolyticus* and *H. influenzae* are haem-auxotrophic.

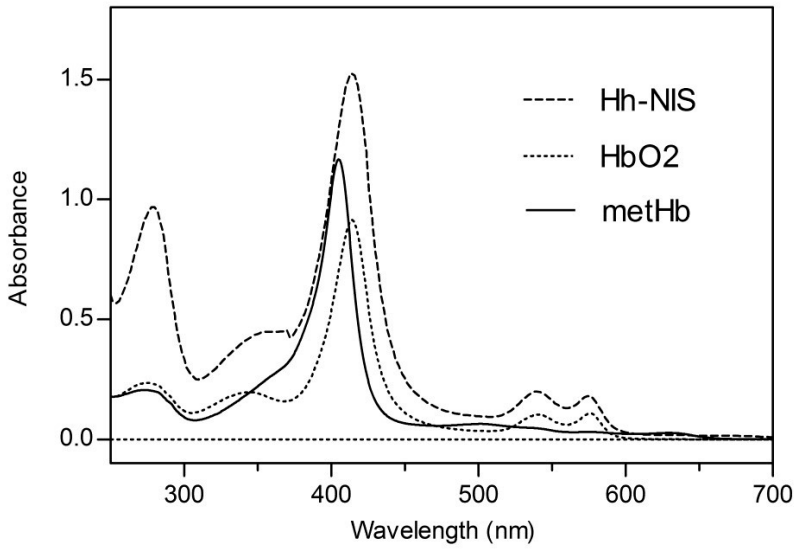

Fig. 7.4. Wavelength scan of recombinant Hh-NIS. The absorbance spectrum of purified recombinant Hh-NIS contained 414 nm, 541 nm, and 576 nm peaks that were similar to absorption peaks in a spectrum of oxy-haemoglobin (HbO2; spectrum provided by D. Gell), but not present in met-haemoglobin (metHb; spectrum provided by D. Gell).

The NIS activity of the apo and holo fractions of recombinant Hh-NIS was investigated. When tested by agar well diffusion assay, apoprotein concentrations between 2.6µM and 21µM (neat preparation) produced measurable clearing zones (Fig. 7.5), and for the holoprotein, zones were measurable between 4.5µM and 36µM (neat preparation). The relative activity of the apoprotein fraction was 1.7-times that of the holoprotein fraction, based on the concentrations required to produce clearing zones of annular radius 3.5, 4, and 4.5mm (Fig 7.5, 7.6). However, as this is a relatively small difference in activity, and it might also have arisen from difficulties experienced in accurately measuring the concentration of the holoprotein, we considered the

activity of both fractions to be equivalent, and that haem was not a cofactor which activated an enzymes, but that it had some other role.

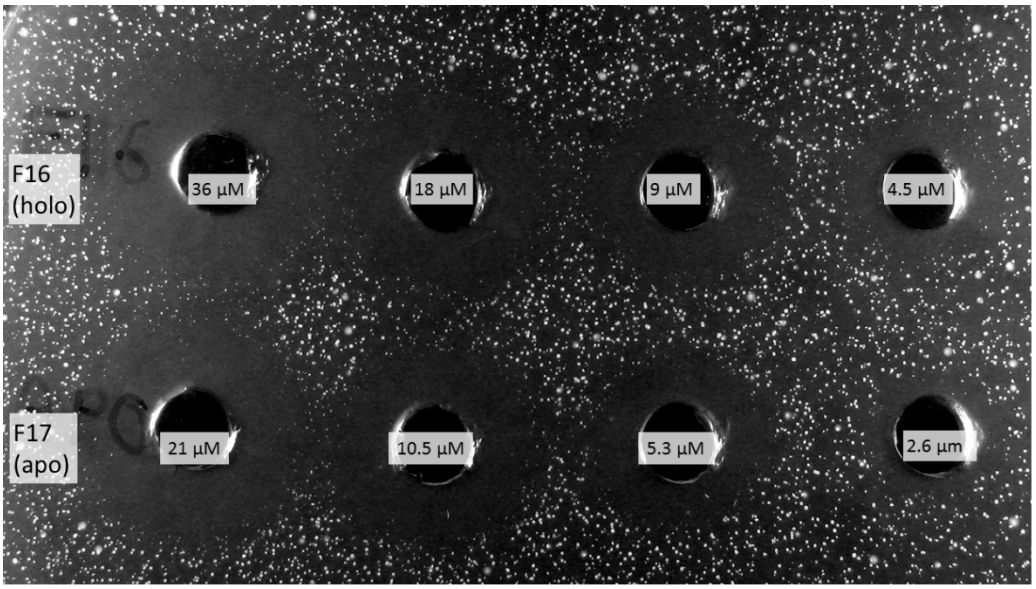

Fig. 7.5. Activity of apo and holo recombinant Hh-NIS fractions. When separated with UnoS1 cation exchange column, and tested with an agar well diffusion assay, there was activity in both F16 (holo) and F17 (apo) fractions.

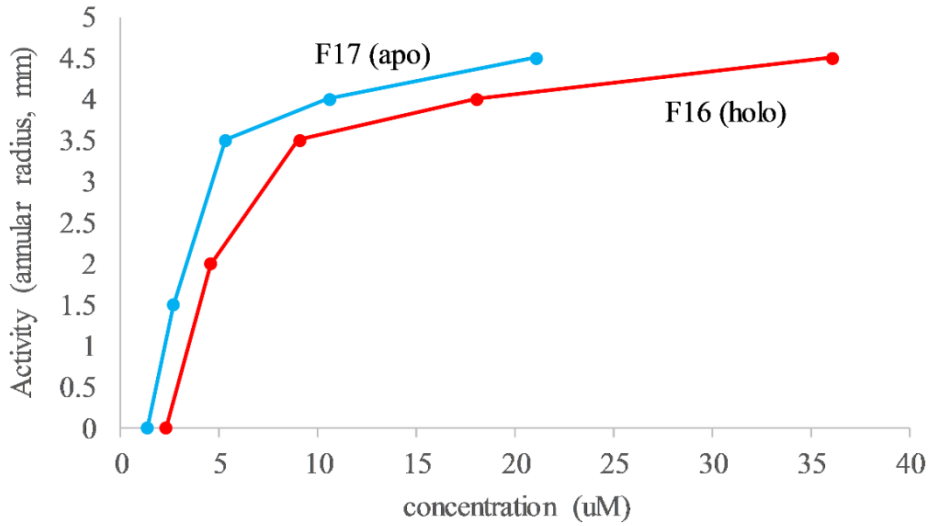

Fig 7.6. NIS activity in apo and holo fractions from recombinant Hh-NIS. The apo fraction (blue line) had consistently more activity than the holo fraction (red line).

The minimum inhibitory concentration of the apo and holo fractions was investigated in haemin-replete broth culture. The holoprotein fraction of recombinant Hh-NIS did not inhibit the growth of NTHi strains NCTC 4560 and NCTC 11315 at any concentration up to 1.7µM (Fig. 7.7). For the apoprotein fraction, there was no inhibition of NTHi strain NCTC 4560 at any concentration, up to 1.0µM (Fig. 7.8). Although the growth of NTHi strain NCTC 11315 was reduced by 30% (from 0.23 to 0.16, OD_{600}) at the lowest apoprotein concentration (0.0001µM), there was no further reduction with increasing concentration. Accordingly, we assumed there to be an absence of inhibitory activity in the apoprotein and the holoprotein fractions, in the haemin-replete conditions of the assay. We then endeavoured to determine the activity of Hh-NIS in haemin-limited conditions.

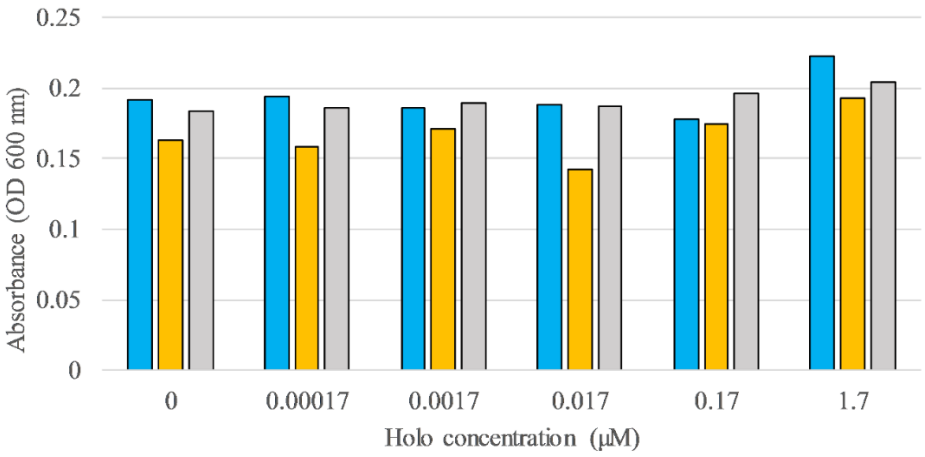

Fig 7.7. Minimum inhibitory concentration (MIC) testing, holo fraction. There was no apparent inhibition of growth of NTHi strains NCTC 4560 (blue), NCTC 11315 (orange) and BW1 (grey) by the holo fraction of recombinant Hh-NIS. Broth is sTSB, which contains 15 µg/mL haemin.

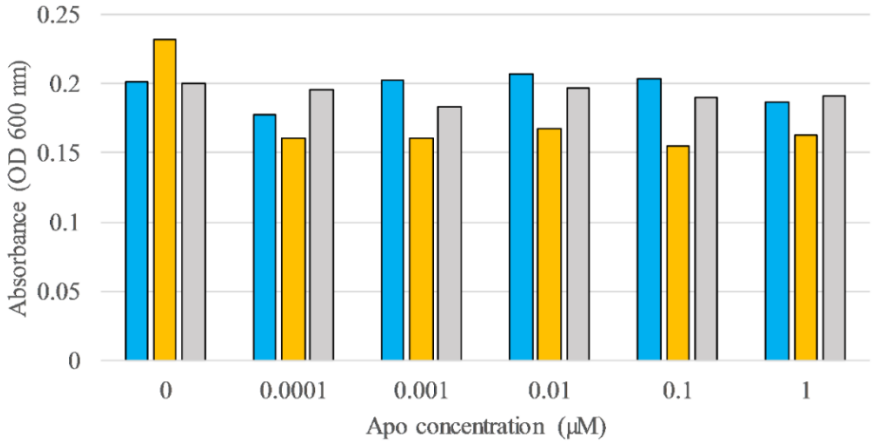

Fig 7.8. MIC testing, apo fraction. There was no apparent inhibition of growth of NTHi strains NCTC 4560 (blue) and NCTC 11315 (orange), and BW1 (grey) by the apoprotein of recombinant Hh-NIS. Broth is sTSB, which contains 15 µg/mL haemin.

For the determination of haemin-limited conditions, the haemin requirements for the growth of BW1 and NTHi strain NCTC 4560 were investigated, and BW1 was found to have a far higher requirement. The maximum absorbance (OD_{600}) for NCTC 4560 (0.28) was attained in only 0.625 µg/mL haemin (Fig. 7.9), whereas the maximum absorbance for BW1 (0.37) required a 25-fold higher concentration (40 µg/mL, Fig. 7.10).

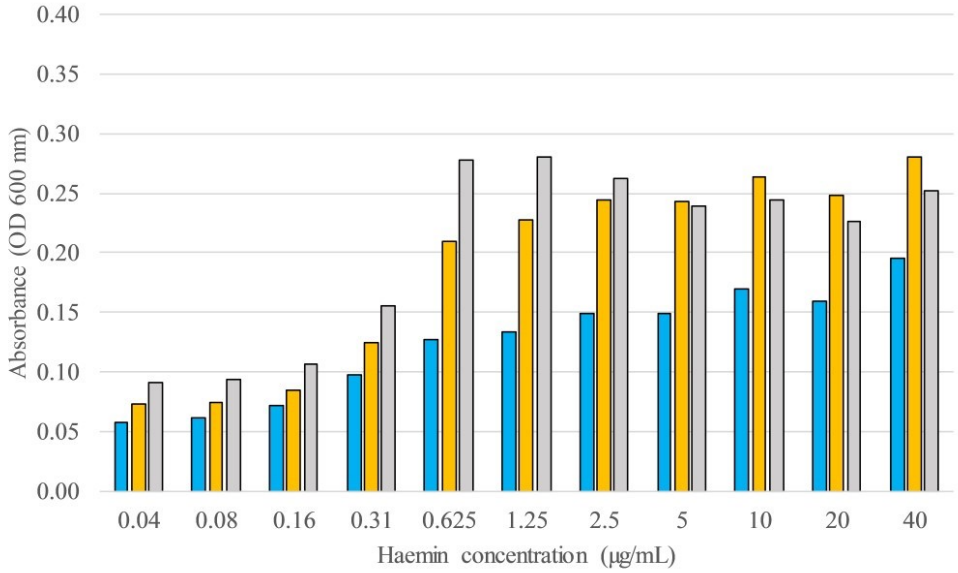

Fig 7.9. Growth response of NTHi to haemin. At 19 hours (grey), the growth of NCTC 4560 was reduced only when the haemin concentrations was 0.31 µg/mL or less. 8 hours (blue), 12 hours (orange). Broth culture in TSB supplemented with Vitox, NAD and variable haemin concentration. Initial absorbance ~0.05 (OD_{600}).

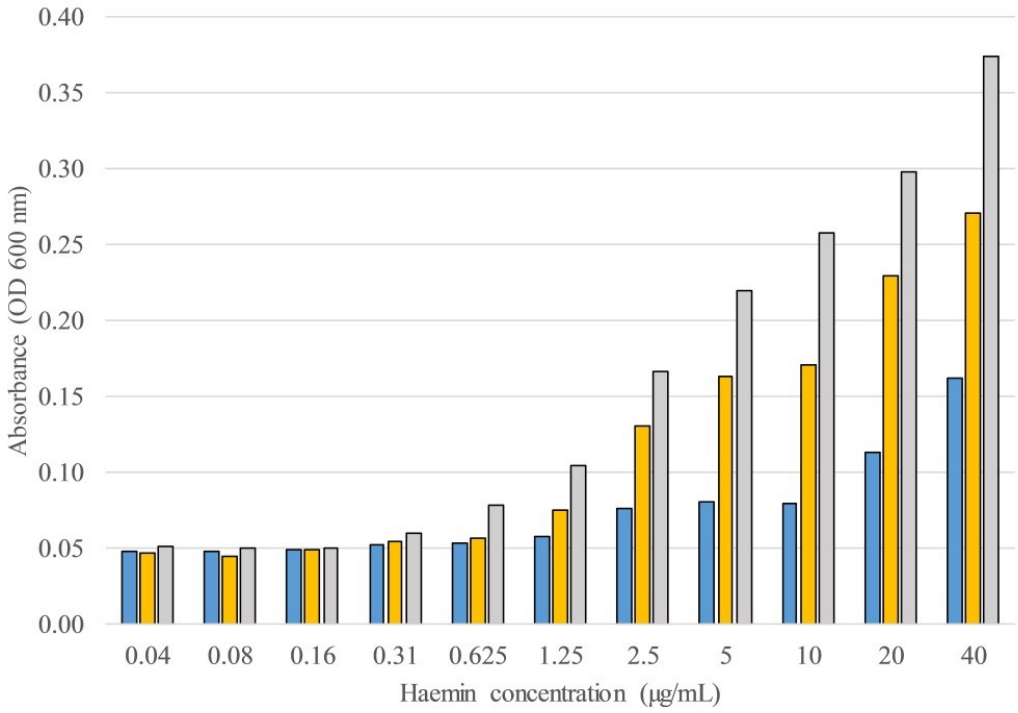

Fig 7.10. Growth response of BW1 to haemin. A haemin concentration of at least 0.31 µg/mL was required for any growth of BW1, and greater concentrations incrementally increased growth. 8 hours (blue), 12 hours (orange), 19 hours (grey). Broth culture in TSB supplemented with vitox, NAD and variable haemin concentration. Initial absorbance ~0.05 (OD_{600}).

The effect of haemin concentration on Hh-NIS activity was then examined, in haemin-limited conditions. Haemin was serially diluted and mixed with BW1-NIS, then activity against NTHi strains NCTC 4560 and NCTC 11315 assessed by agar well diffusion assay. With haemin concentrations of 40 µg/mL and 20 µg/mL, the activity of BW1-NIS was completely neutralised (Fig. 7.11, 7.12), and, even increased the size of the surrounding colonies (Fig. 7.11, only NCTC 11315 shown). At 10 µg/mL there was slight inhibitory activity, and with haemin concentrations ranging from 2.5 µg/mL to 0.16 µg/mL, inhibitory activity was clearly observed and unchanged. With the results from agar well diffusion assay showing that a

reduced haemin concentration was critical, the activity of Hh-NIS in broth could then be investigated.

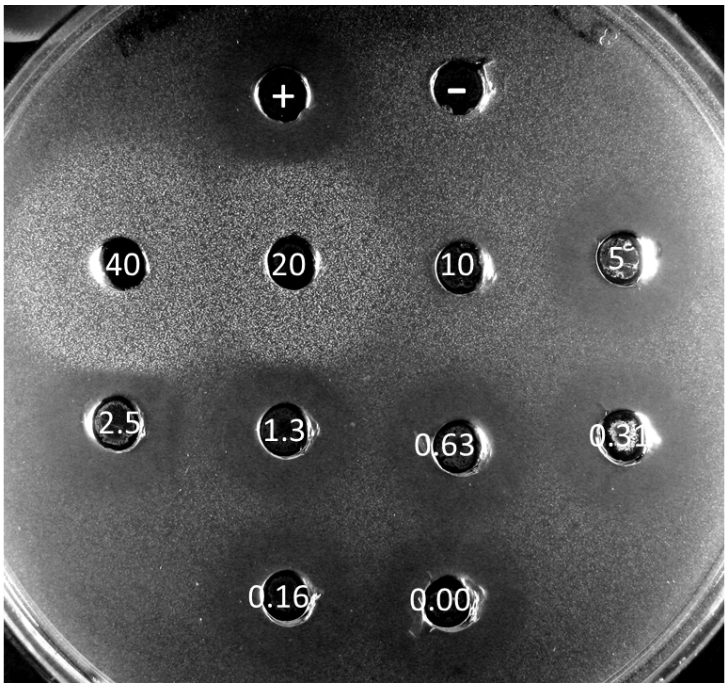

Fig 7.11. Addition of haemin neutralises the inhibitory activity of BW1-NIS. Agar well diffusion assay with wells containing BW1-NIS mixed with haemin at variable concentrations (values shown, µg/mL). Positive control (+) contains BW1-NIS only. Negative control (-) contains DPBS. Indicator is NTHi strain NCTC 11315.

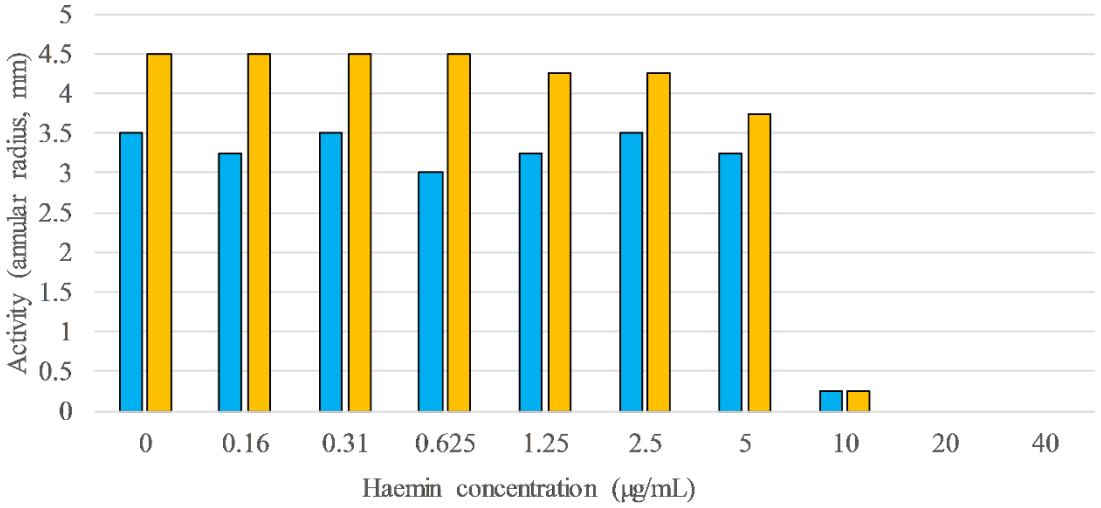

Fig 7.12. Addition of haemin neutralises the inhibitory activity of BW1-NIS. Agar well diffusion assay, with NCTC 4560 (blue) and NCTC 11315 (orange) as indicators.

The effect of Hh-NIS on NTHi and BW1 was investigated in broth, using haemin-limited conditions. With haemin at 0.625 µg/mL and 5 µg/mL (7.7µM), addition of 7.7µM of the holoprotein fraction of recombinant Hh-NIS resulted in reduced growth of NCTC 4560 (Fig. 7.13) and increased growth of BW1 (Fig. 7.14), with the effect more obvious at the lower haemin concentration (Fig 7.13, 7.14, blue lines). Although preferable to similarly examine the apoprotein fraction, only the holoprotein fraction was available at the time of testing.

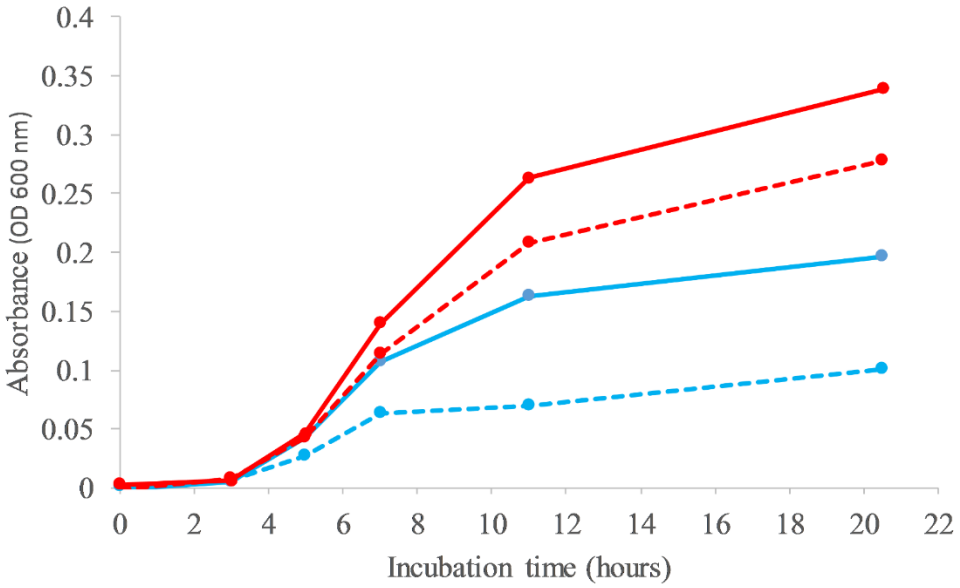

Fig 7.13. In haemin-limited broth culture, the holo fraction of recombinant Hh-NIS reduced the growth of NTHi. With haemin at 5 µg/mL (red) and 0.625 µg/mL (blue), the addition of the Hh-NIS preparation (broken line) reduced the growth of NTHi strain NCTC 4560, compared to the condition with no Hh-NIS (solid line). The base media was TSB supplemented with Vitox and NAD.

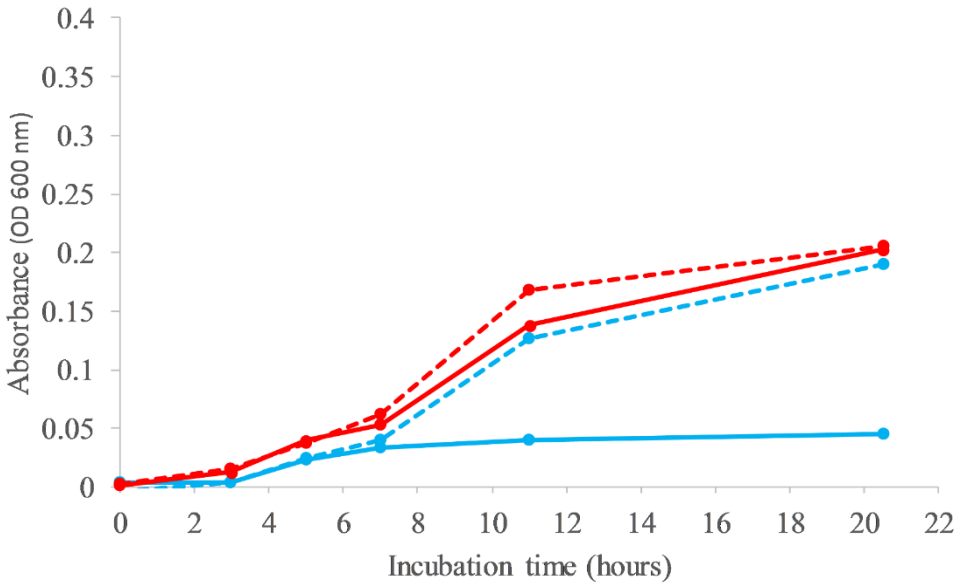

Fig 7.14. In haemin-limited broth culture, the holo fraction of recombinant Hh-NIS increased the growth of BW1. With haemin at 5 µg/mL (red) and 0.625 µg/mL (blue), the addition of the Hh-NIS (broken line) increased the growth of *H. haemolyticus* isolate BW1, compared to the condition with no Hh-NIS (solid line). The base media was TSB supplemented with vitox and NAD.

7.1 Discussion

Together, the evidence we have fit a model for the mechanism of action of Hh-NIS, as follows: a minority of isolates of *H. haemolyticus* secrete Hh-NIS, which is a haemophore that binds haem and makes it available for use by *H. haemolyticus* isolates which produce Hh-NIS, but unavailable for use by NTHi.

The recombinant Hh-NIS was separated into holoprotein and apoprotein fractions, which were similar in activity. This was an unexpected result, as the holoprotein was 13-times more abundant in haem, and haem is typically a co-factor. This suggests that haem is not a co-factor which makes the apoprotein active, but that it has a different role.

In the haemin-replete broth culture, the holoprotein and apoprotein fractions of recombinant Hh-NIS were not inhibitory to the growth of NTHi (Fig. 7.7, 7. 8). Possibly this result was due to the concentrations of the Hh-NIS preparations that were used. In the broth, because of limited

material availability, the highest concentrations which could be achieved were 1.0µM (apoprotein) and 1.7µM (holoprotein), both less than required to produce clearing zone in the agar well diffusion assay (Fig. 7.6). A repeat of this experiment, but with much higher concentrations of the apo and holo fractions, would help to test our model for the mechanism of activity of Hh-NIS.

The effect of haemin addition to wells in an agar well diffusion assay was to neutralise the activity of Hh-NIS (Fig. 7.11). This result explained idiosyncrasies encountered in the development of the agar well diffusion assay, whereby reliable formation of clearing zones required the growth media to be at half-strength and aged several days, and the zone sizes also increased with the age of the media. With growth media at half-strength, the haemin will also be at half-strength, which will promote the formation of clearing zones, and with extended storage, the zone sizes increase because haemin is unstable in aqueous solution.

The final evidence we had for describing the mechanism of action of Hh-NIS, is that in haemin-limited broth culture, the holoprotein inhibited the growth of NTHi, and promoted the growth of BW1 (Fig. 7.13, 7.14). Although the inhibition of NTHi confirmed the results in agar well diffusion assay, the promotion of BW1 growth was informative for the development of our model, as we had not observed this previously.

For the overall aim of this project, the prevalence of haem acquisition genes in NTHi has been linked with their pathogenic potential, which could have implications for the usefulness of Hh-NIS-containing *H. haemolyticus* isolates as probiotics. In a study of the relationship between pathogenicity and haem-acquisition genes, the prevalence of *hxuA, hxuB, hxuC, hemR,* and *hup* in NTHi, was shown to be much higher than in *H. haemolyticus* (Hariadi et al., 2015). Also in the same study, the prevalence of *hxuA, hxuB, hxuC,* and *hemR* was higher in NTHi isolates

from middle-ear acute otitis media samples, than in NTHi from throats swabs of healthy children. These results highlight the possibility that Hh-NIS may be a virulence factor for *H. haemolyticus*, which is also a rare cause of invasive disease in the immunocompromised. We suggest that a study of these haem acquisition genes, including *Hh-NIS*, in invasive, non-invasive, and Hh-NIS producing isolates of *H. haemolyticus* would be informative with regards to an understanding of the virulence factors of invasive strains of *H. haemolyticus*, and the role of Hh-NIS. A comparison with haemophores of other pathogens may also be informative with regards to the pathogenic potential of Hh-NIS producing *H. haemolyticus*.

A haemophore with functional similarities to Hh-NIS, exists in other opportunistic pathogens, but not *H. influenzae*, *H. haemolyticus* or other human-obligate pathogens. *Serratia marcescens, Pseudomonas aeruginosa* and *Yersinia pestis* secrete HasA, which binds haem and delivers it to the outer-membrane receptor, HasR, for transport across the outer membrane (Ghigo et al., 1997; Letoffe et al., 1994; Létoffé et al., 1998; Rossi et al., 2001). Like Hh-NIS, HasA binds free haem (Létoffé et al., 1999; Yukl et al., 2010), which differs markedly from the HxuA haemophore of *H. influenzae*, that binds to the haemopexin-haem complex, resulting in the release of the haem (Fournier et al., 2011; Hanson et al., 1992), and is associated with pathogenicity (Hariadi et al., 2015; Morton et al., 2007). As such, although Hh-NIS is a haemophore, we do not expect that it will be a virulence factor.

In conclusion, Hh-NIS appears to be a haemophore, secreted by some isolates of *H. haemolyticus*, and binds haem into a form which is available to the same *H. haemolyticus* isolates, but is then no longer available to NTHi, resulting in inhibition of the growth of NTHi, but only under haem-limited conditions. Also, although Hh-NIS does appear to be a haemophore, we do not expect that it is a virulence factor, as it has similarities with HasA, which is not associated with pathogenicity.

Chapter 8.

General Discussion

The overall aim of this PhD project was to investigate the potential of *H. haemolyticus* as a respiratory tract probiotic against NTHi infection, with a focus on secreted NTHi inhibiting substances (NIS). A protein with NIS activity (Hh-NIS, accession EGT80255), produced by *H. haemolyticus* isolates BW1 and RHH122, was identified by comparison to a whole genome sequence of *H. haemolyticus* strain M19107, using mass spectrometry analysis. Hh-NIS was shown to inhibit the growth of NTHi, as well as promote the growth of BW1, in haem-limited conditions. A proposed mechanism for the activity of Hh-NIS was given, whereby Hh-NIS is a haemophore that binds haem into a form available for use by *H. haemolyticus* isolates which produce Hh-NIS, but is unavailable for use by NTHi.

Initially (Chapter 3), SYBR Green RTPCR assays were developed for the identification of *H. haemolyticus* and *H. influenzae*, then used on a collection of isolates. At the time of their publication (Latham et al., 2015), these were the only RTPCR assays we were aware of which positively identified *H. haemolyticus*. SYBR Green was chosen over hydrolysis probes, as these assays are less costly and more readily available. However, subsequently there has been continued interest in *H. haemolyticus* and its interaction with NTHi (de Gier et al., 2015; Pickering et al., 2016), and there are alternative RTPCR assays for *H. haemolyticus* identification, which utilise hydrolysis probes, and target *hypD* (Price et al., 2017) or *purT* (Hu et al., 2016). However, our assays, which target *sodC* and *hpd*, are still the only SYBR Green RTPCR method for *H. haemolyticus* identification we are aware of, apart from a PCR-high-resolution melt method (Pickering et al., 2014) that we found unreliable in our laboratory.

For Chapter 4, *H. haemolyticus* isolates were screened for phenotypic inhibition of NTHi, and the isolates BW1 and RHH122 were identified as consistent producers of a diffusible substance that inhibited NTHi. To describe what we now understand to be the activity of a haemophore, the term “NTHi inhibiting substance” (NIS) was introduced. In a publication about the same

protein (Latham et al., 2017) the term “bacteriocin-like substance” was also used. Most commonly, inhibitory substances, such as bacteriocins and antibiotics, exert their activity directly upon organisms. However, as we now understand this inhibitory activity is due to *H. haemolyticus* producing a substance which deprives NTHi of a commonly required nutrient (haem), the term “inhibitory substance” is likely to be misleading. As a result, we suggest that future publications should avoid referring to this protein as an “inhibitory substance”, “bacteriocin-like substance” or similar, even though the term has been used for consistency throughout this thesis.

In Chapter 6, the amount of Hh-NIS that could be recovered from broth cultures of *Hh-NIS*-positive isolates was highly variable. This was the case even for the six isolates with *Hh-NIS* ORF sequences identical to M19107, with BW1 and RHH122 producing more NIS activity than any, even when adjusted for cell density. Clearly, there are differences among the isolates in how the Hh-NIS is being produced, and as we are suggesting that *H. haemolyticus* isolates which secrete Hh-NIS have a competitive advantage against NTHi, elucidation of the control of Hh-NIS production and secretion is desirable.

Structural homology modelling of Hh-NIS, in Chapter 6, showed it was most closely related to haemoglobin-haptoglobin utilisation protein A (HpuA) in *Neisseria spp*. and *Kingella spp*. Control of HpuA expression at the translational level has been demonstrated in *N. gonohorreae*, with insertional mutations in a poly-G tract in the ORF causing frame shifts, and on/off phase variability (Chen et al., 1998). However, this mechanism is not consistent with the expression variability or the homogeneity of the ORF sequences in the six *H. haemolyticus* isolates. Therefore, we suggest that control of expression at the transcription level is more likely, and that initially the sequences adjacent to the *Hh-NIS* ORF should be investigated, as it is the likely location of a promotor or other genes in an operon which influence Hh-NIS production and

secretion. Later, investigations of the involvement of other loci may be required, as the mechanisms which govern the concentration of Hh-NIS in the surrounding environment may not be fully under the control of an Hh-NIS operon.

In Chapter 7, the possibility that Hh-NIS is a virulence factor of *H. haemolyticus* was discussed, but then considered unlikely, due to its apparent similarity to HasA, and dissimilarity with HxuA. Nevertheless, as we are suggesting the deliberate introduction into humans of *H. haemolyticus* strains which produce Hh-NIS, we advise caution, considering the *Hh-NIS* ORF was present in two of three invasive *H. haemolyticus* isolates, and all of the three genome sequences of *H. quentini*. As a result, prior to human trials, we recommend completion of the study of haem-acquisition genes in *H. haemolyticus* that was suggested in Chapter 7, as well as *in vivo* testing of the pathogenicity of isolates with and without *Hh-NIS*.

Optimally, to understand the significance of a gene, knockout mutants are made and tested in biologically relevant conditions, as described in the molecular Koch's postulates (Falkow, 1988) for the determination of genes as virulence factors. Due to the constraints of the project, and as a substitute for an over-expression or gene knock-in experiment, purified Hh-NIS preparations were added to broth cultures of BW1 and NTHi, the results of which showed the Hh-NIS inhibited NTHi and promoted BW1 growth, but only in haemin-limited conditions. Although these experiments were informative, they do not substitute for studies with knockout and knock-in mutants, which isolate the effects of the Hh-NIS. As a result, we suggest that for any further investigation of the biological significance of Hh-NIS, the priority is to make knockout and knock-in mutants, and test them in conditions which mimic those present in the host.

On a more fundamental level, in this project we have made the initial discovery of a protein made by *H. haemolyticus*, which appears to be a haemophore, but that has some structural similarities to HpuA, a haemoglobin receptor of *Neisseria spp*. and *Kingella spp*. However, unlike HpuA, there are still many discoveries about Hh-NIS that are yet to be made, and which provide future research directions; tertiary and quaternary structure, manufacture and release from the bacterial cell, interacts with any haem-protein complexes, the strength and location of the haem, the involvement of any cell-surface receptors, and translocation of the bound ligand into cell.

Finally, and with regards to the overall aim of this project, we have discovered *H. haemolyticus* isolates which were inhibitory to the growth of NTHi *in vitro*. This discovery provides evidence that microbial interference may be a viable mechanism for the inhibition of NTHi colonisation of the upper respiratory tract, and furthermore, that *H. haemolyticus* strains expressing Hh-NIS may be suitable as probiotics in this application. Accordingly, in the absence of an effective vaccine for the control of NTHi, for the development of a microbial interference approach, we recommend *in vivo* trials in humans. However, as haem-acquisition genes are associated with pathogenicity, in these trials a cautionary approach is advised.

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Appendix 1.

NTHi tested for production of NIS. NTHi indicator strains were NCTC 11315 and NCTC. Testing by agar well diffusion assay, with enriched cell-free culture broths. No isolates produced a NIS.

CF28A	M19	RHH50
CF29	M21	RHH51
CF31	M27	RHH53
CF34	NF3	RHH54
CF48	NF30	RHH55
Ci11	NF37	RHH56
Ci2	NF38	RHH60
Ci29	NF7	RHH62
Ci3	RHH1	RHH64
Ci30	RHH12	RHH66
Ci34	RHH14	RHH68
Ci37	RHH15	RHH7
Ci5	RHH17	RHH73
Ci51	RHH19	RHH74
Ci8	RHH20	RHH8
Ci9	RHH21	RHH80
H62	RHH22	RHH81
L1	RHH28	RHH82
L10	RHH29	RHH83
L13	RHH3	RHH84
L139	RHH31	RHH85
L143	RHH32	RHH88
L146	RHH34	RHH89
L15	RHH35	RHH9
L21	RHH36	RHH90
L24	RHH37	ATCC 49766
L26	RHH38	NCTC 4560
L267	RHH39	NCTC 11315
L27	RHH4	
L35	RHH41A	
L44	RHH41B	
L60	RHH42	
L76	RHH43	
L79	RHH44	
M03	RHH46	
M07	RHH47	
M11	RHH48	

Appendix 2.

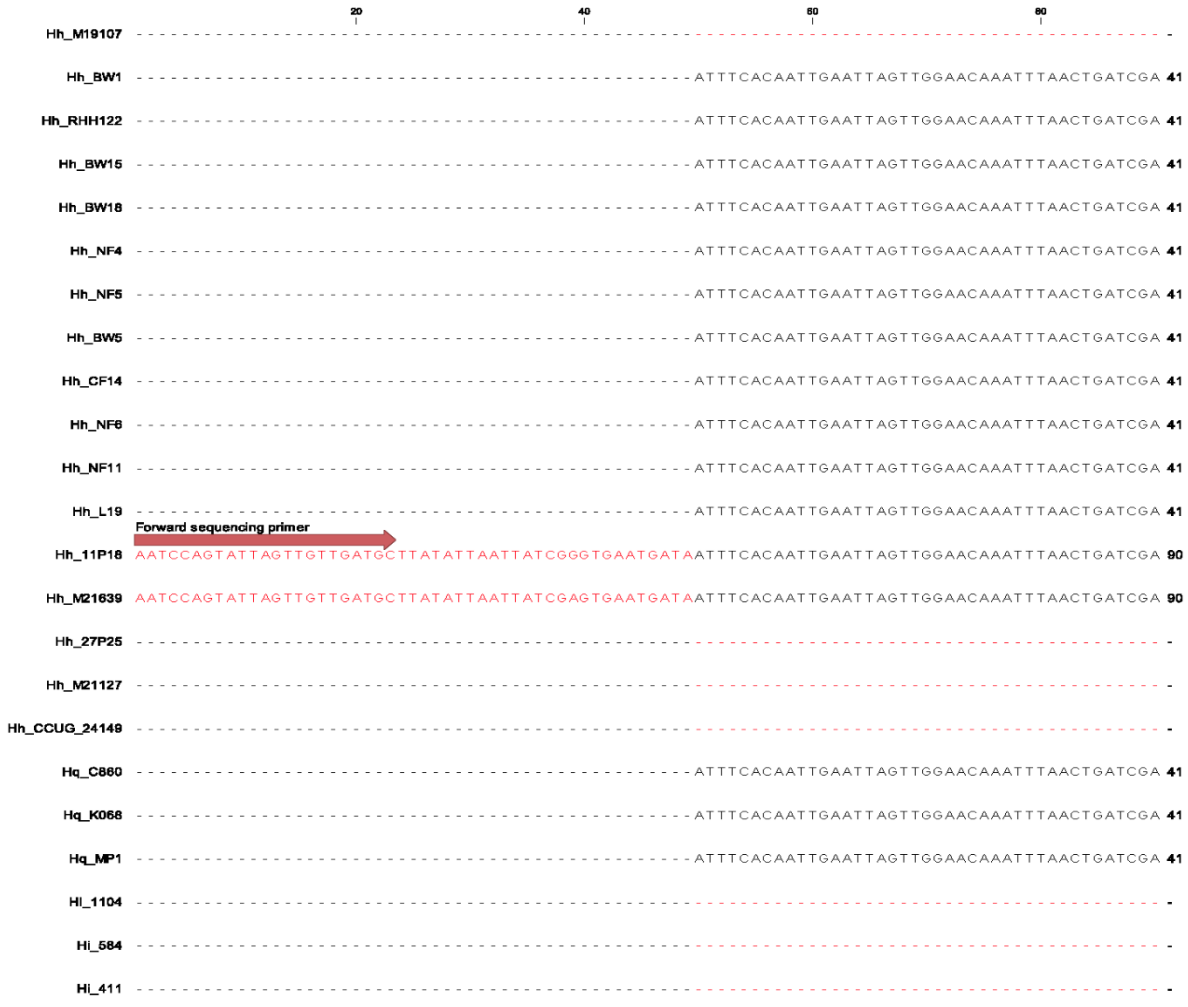
Susceptibility to BW1-NIS and haemocin. Isolates/strains used as indicators in agar well diffusion assay. + susceptible, - not susceptible.

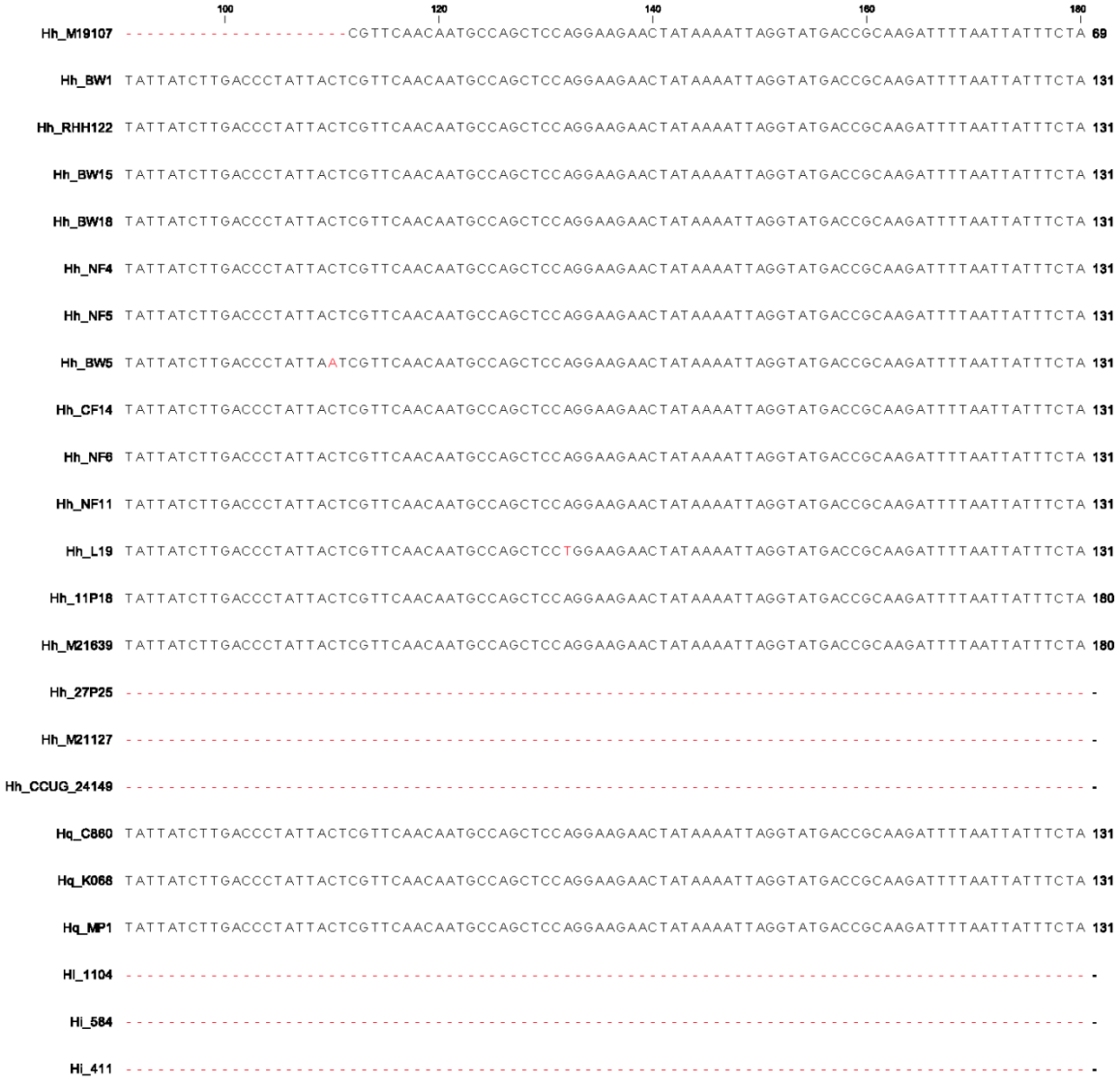
Strain	Species	BW1-NIS	Haemocin		Strain	Species	BW1-NIS	Haemocin
ATCC 9007	Hi	+	+		L15	Hi	+	-
ATCC 43163	Hi	+	-		L19	Hh	-	+
ATCC 10211	Hi	+	-		L21	Hi	+	+
NCTC 4560	Hi	+	+		L23	Hh	-	-
NCTC 11315	Hi	+	+		L35	Hi	+	+
ATCC 49766	Hi	+	+		L37	Hh	+	+
ATCC 33390	Hh	-	+		L43	Hh	+	+
86	Hi	+	+		L48	Hh	+	+
RA	Hi	+	+		L52	Hh	-	-
RD	Hi	+	+		L66	Hh	+	+
MDU3	Hi	+	-		L71	Hh	-	-
BW1	Hh	-	+		L76	Hi	+	+
BW2	Hh	+	+		L79	Hi	+	+
BW15	Hh	+	-		L93	Hh	-	-
BW16	Hh	+	+		L95	Hh	+	+
BW18	Hh	+	+		L139	Hi	+	+
CF5	Hh	+	+		L143	Hi	+	+
CF14	Hh	+	-		L146	Hi	+	+
CF26	Hh	+	+		L267	Hi	+	+
CF28A	Hi	+	+		M05	Hh	+	+
CF28B	Hh	+	-		M26	Hh	+	+
CF31	Hi	+	+		NF1	Hh	+	+
CF34	Hi	+	+		NF3	Hi	+	+
CF48	Hi	+	+		NF7	Hi	+	+
CF53	Hh	-	-		NF30	Hi	+	+
Ci2	Hi	+	+		NF37	Hi	+	+
Ci3	Hi	+	+		NF38	Hi	+	+
Ci5	Hi	+	+		RHH4	Hi	+	+
Ci8	Hi	+	+		RHH7	Hi	+	+
Ci9	Hi	+	+		RHH12	Hi	+	+
Ci29	Hi	+	+		RHH14	Hi	+	+
Ci37	Hi	+	+		RHH15	Hi	+	+
L4	Hh	+	+		RHH17	Hi	+	+
L5	Hh	-	-		RHH19	Hi	+	+
L6	Hh	-	-		RHH21	Hi	+	+
L13	Hi	+	+		RHH32	Hi	+	+

Strain	Specie	BW1-	Haemoci		Species, strain	BW1-	Haemoci
RHH41	Hi	+	+		S. pneum, 10582	-	-
RHH41	Hi	+	+		S. pneum, 6305	-	-
RHH43	Hi	+	+		S. pyogenes, 19615	-	-
RHH44	Hi	+	+		S. bovis, 35034	-	-
RHH47	Hi	+	+		E. faecalis, 29212	-	-
RHH48	Hi	+	+		E. faecalis, POW 1995	-	-
RHH50	Hi	+	+		E. faecicum, 99p51661	-	-
RHH51	Hi	+	+		E. gallinarum, ATCC 49673	-	-
RHH56	Hi	+	+		A. baumannii, ATCC …	-	-
RHH58	Hh	-	-		Burk. cepacia, ATCC	-	-
RHH60	Hi	+	+		C. albic., ATCC 10231	-	-
RHH61	Hh	-	-		E.coli, DH5 alpha	-	+
RHH62	Hi	-	+		E.coli, NCTC 11560	-	+
RHH64	Hi	+	+		E.coli, 10418	-	+
RHH68	Hi	+	+		Proteus vulgaris, ATCC	-	-
RHH73	Hi	+	+		M. cat., ATCC 25238	-	+
RHH75	Hh	+	+		N. lact., ATCC 239709	-	+
RHH80	Hi	-	+		P. aeruginosa, PA01	-	-
RHH82	Hi	+	+		MRSA, ATCC 33591	-	-
RHH84	Hi	+	+		S. epid., ATCC 12228	-	-
RHH88	Hi	+	+		S. aureus, ATCC 25923	-	-
RHH89	Hi	+	+		S. aureus, ATCC 29213	-	-
RHH91	Hi	+	+				
RHH92	Hi	+	+				
RHH93	Hi	+	+				
RHH96	Hi	+	+				
RHH99	Hi	+	+				
RHH101	Hi	+	+				
RHH103	Hi	+	+				
RHH106	Hi	+	+				
RHH112	Hh	-	-				
RHH116	Hi	+	+				
RHH118	Hi	+	+				
RHH125	Hi	+	+				
RHH126	Hi	+	+				
RHH127	Hi	+	+				

Appendix 3.

Alignment of Hh-NIS sequences.

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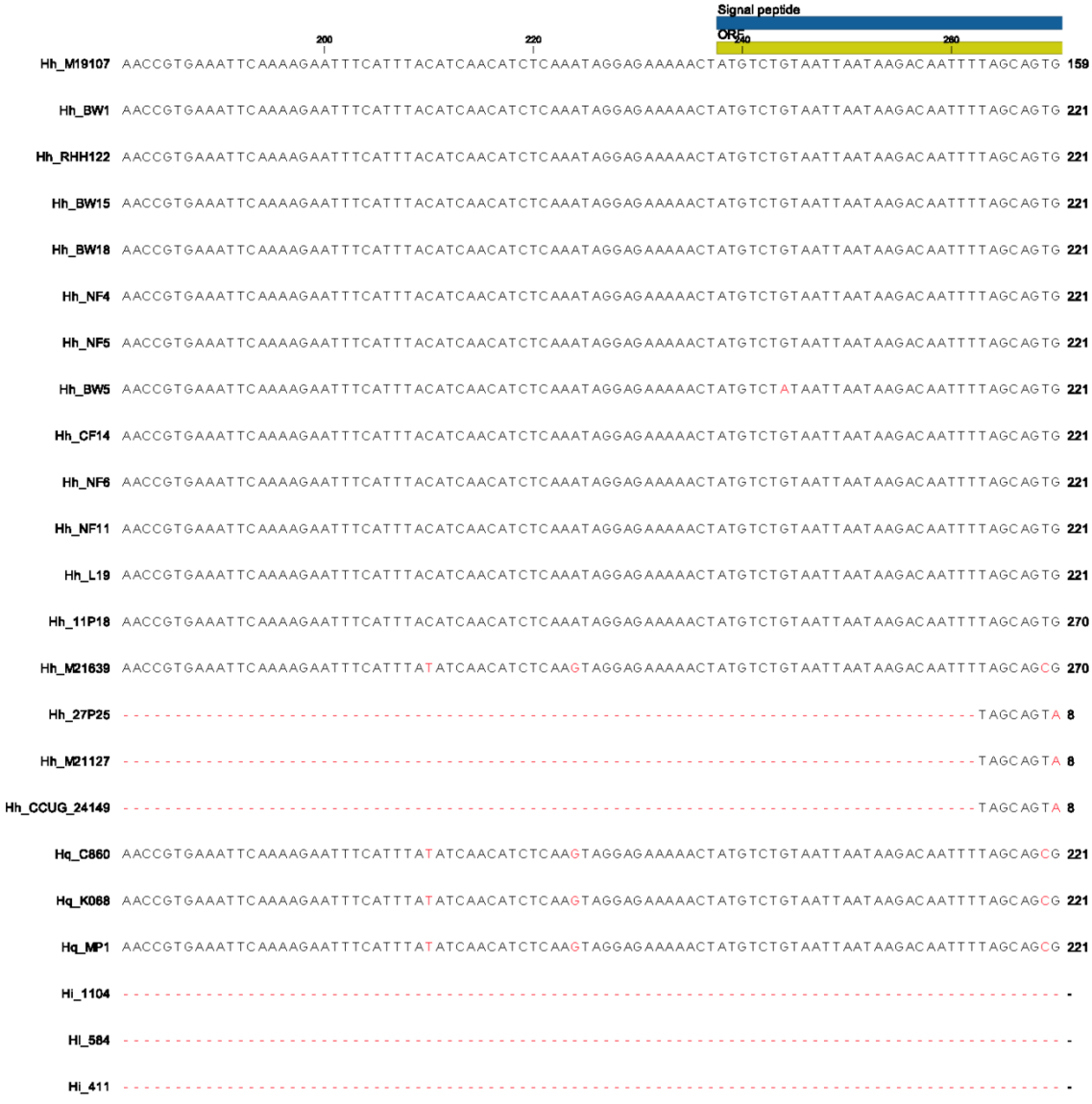
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Hi_1104 ---------- -
HI_584 ---------- -
Hi_411 ---------- -


Signal peptide
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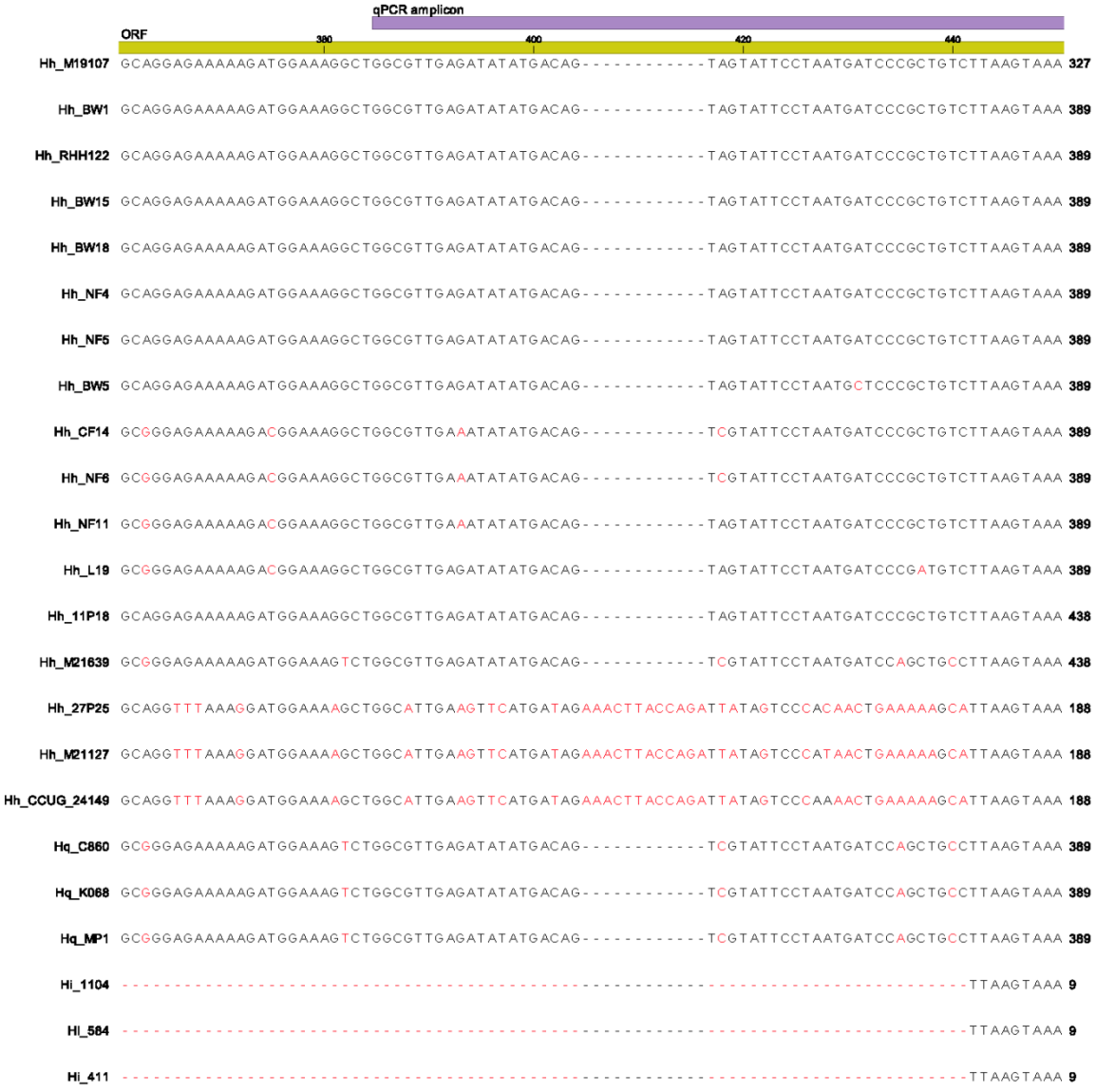
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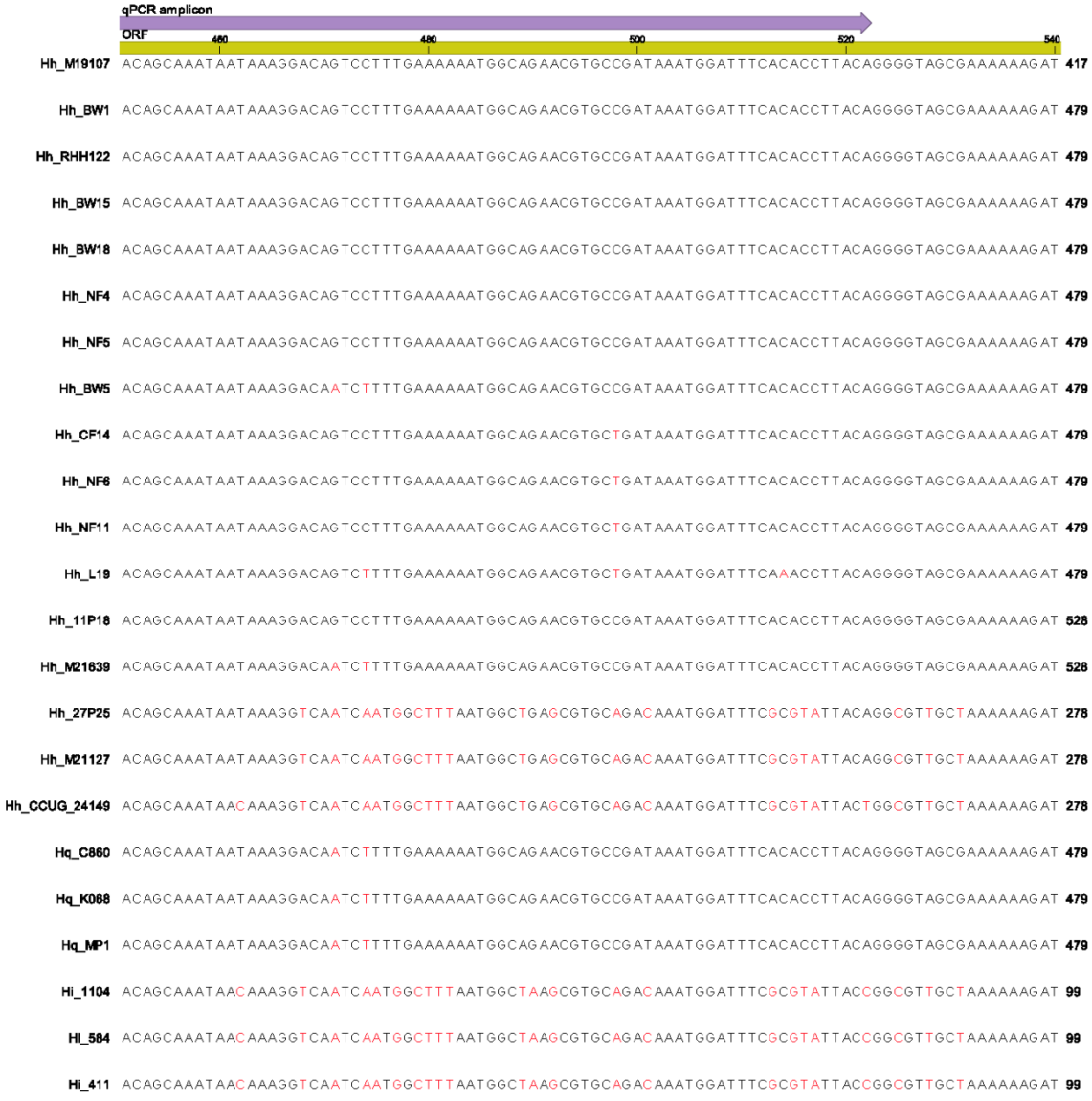
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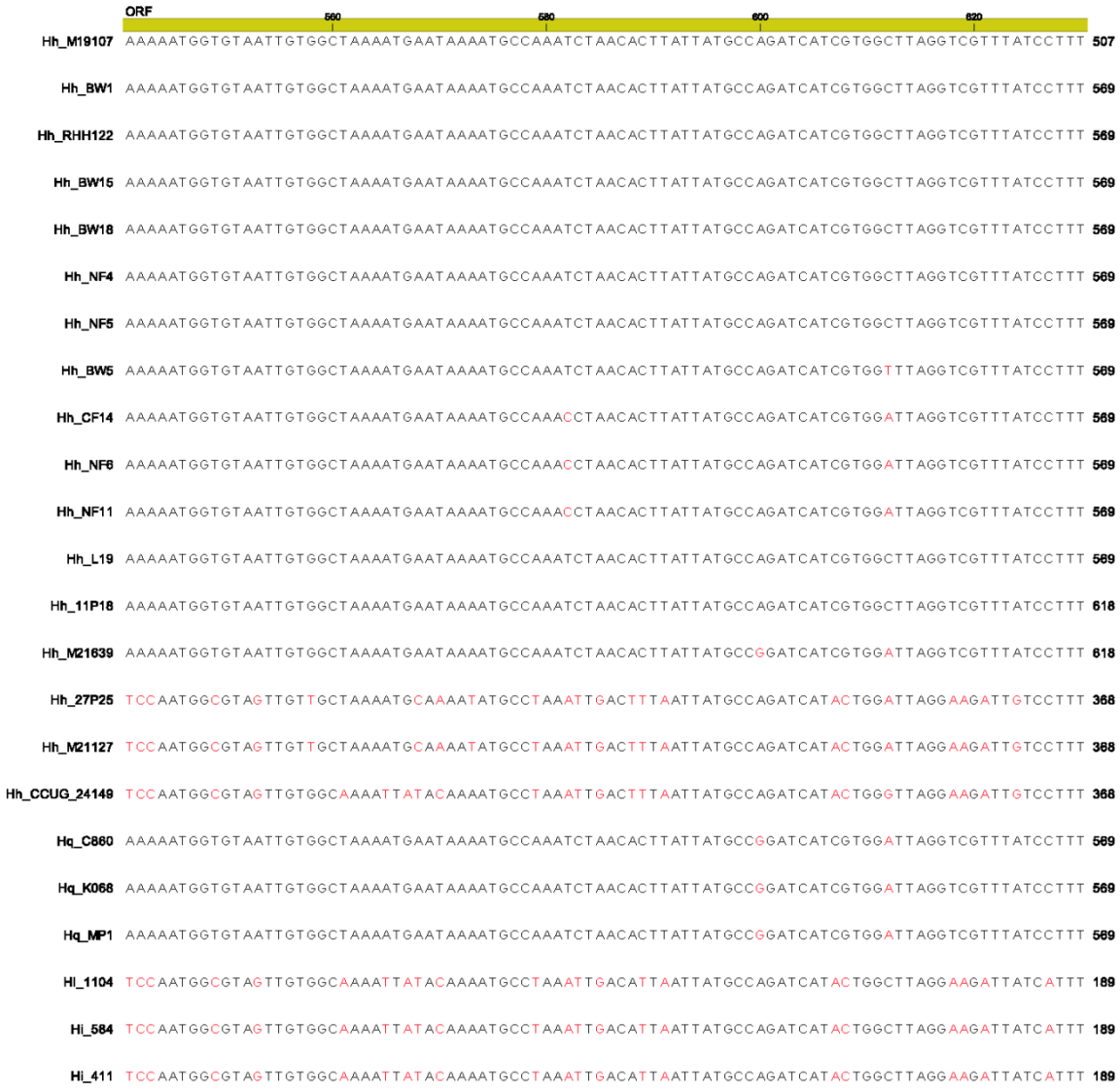
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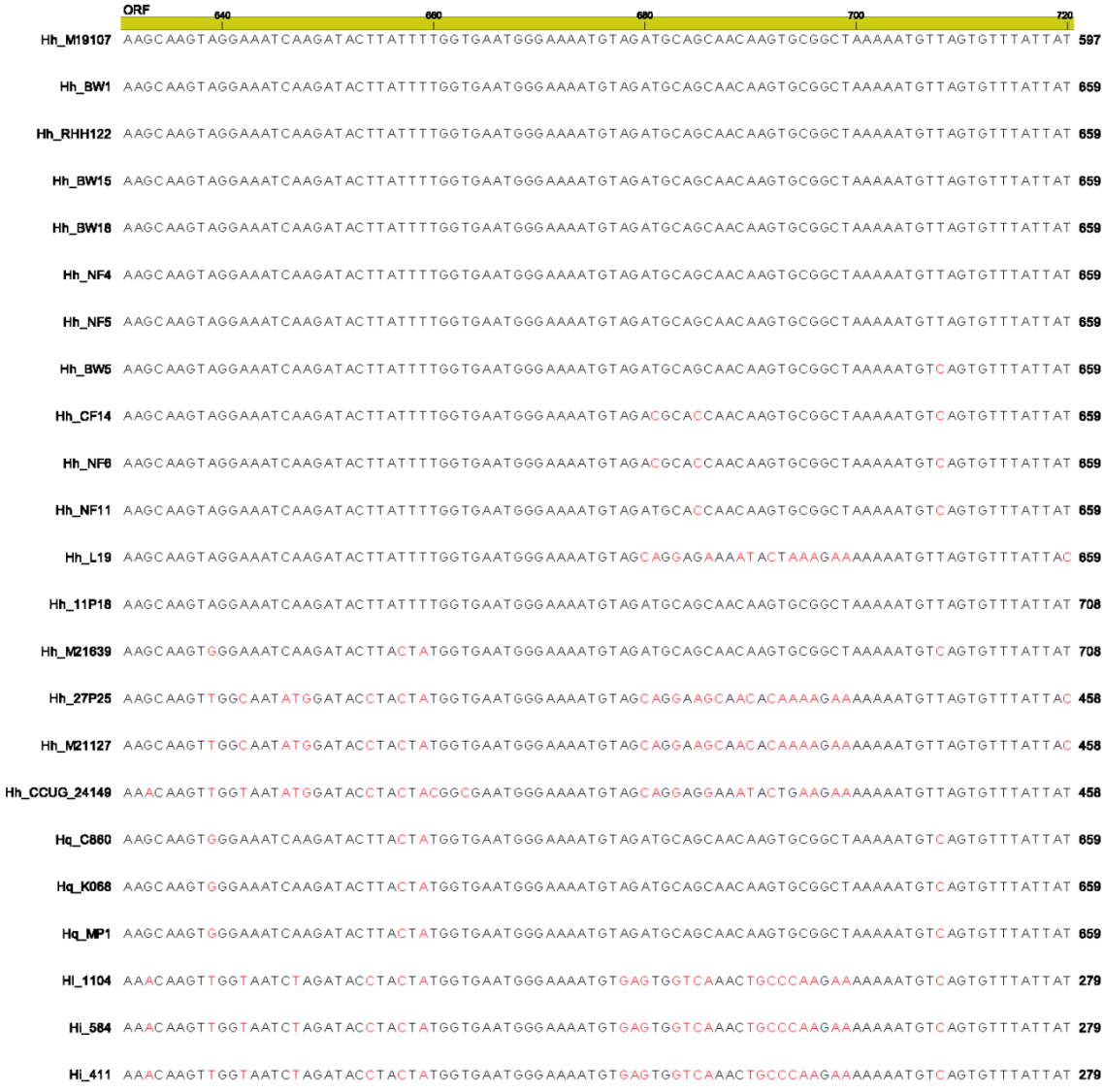
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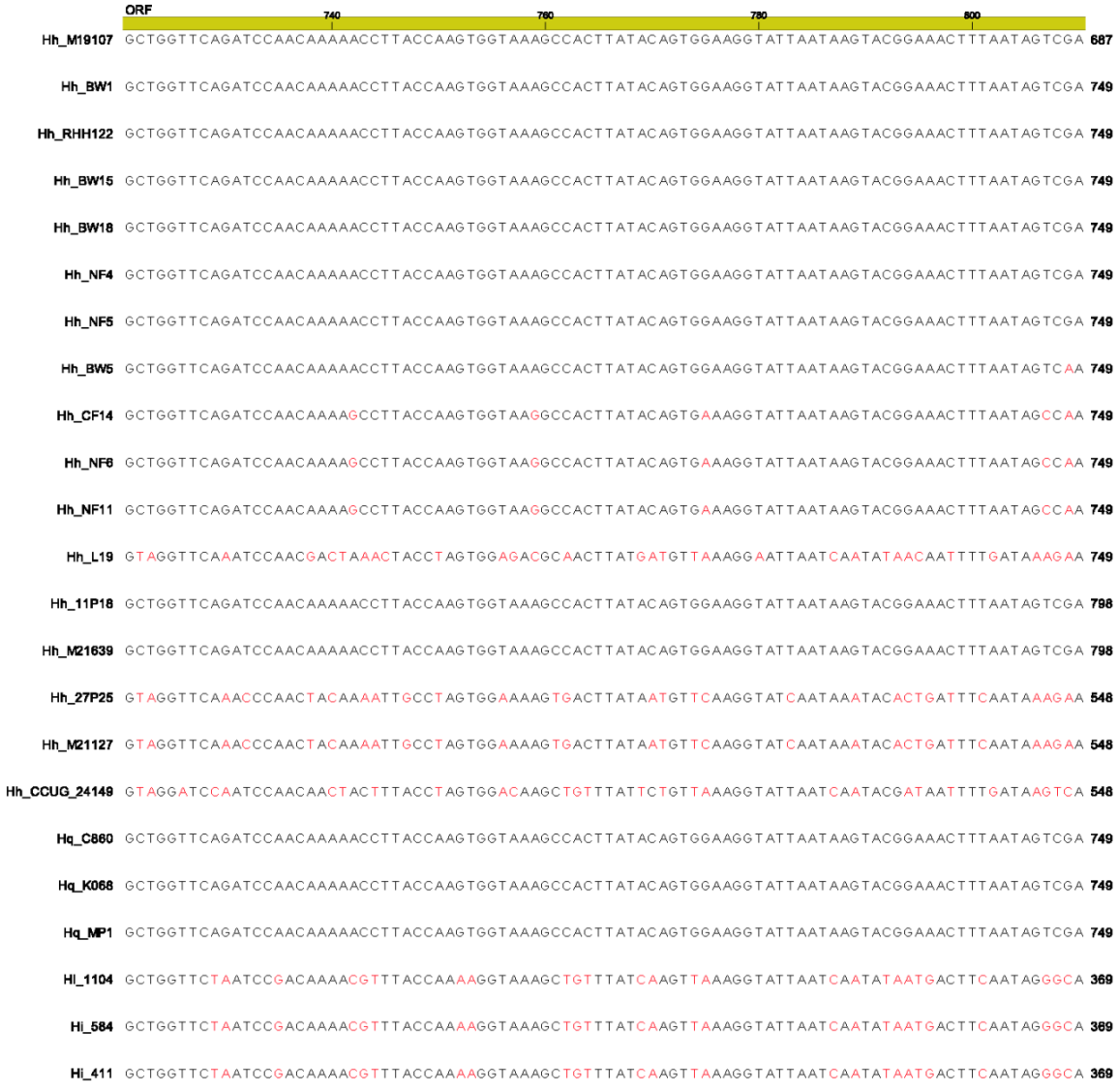
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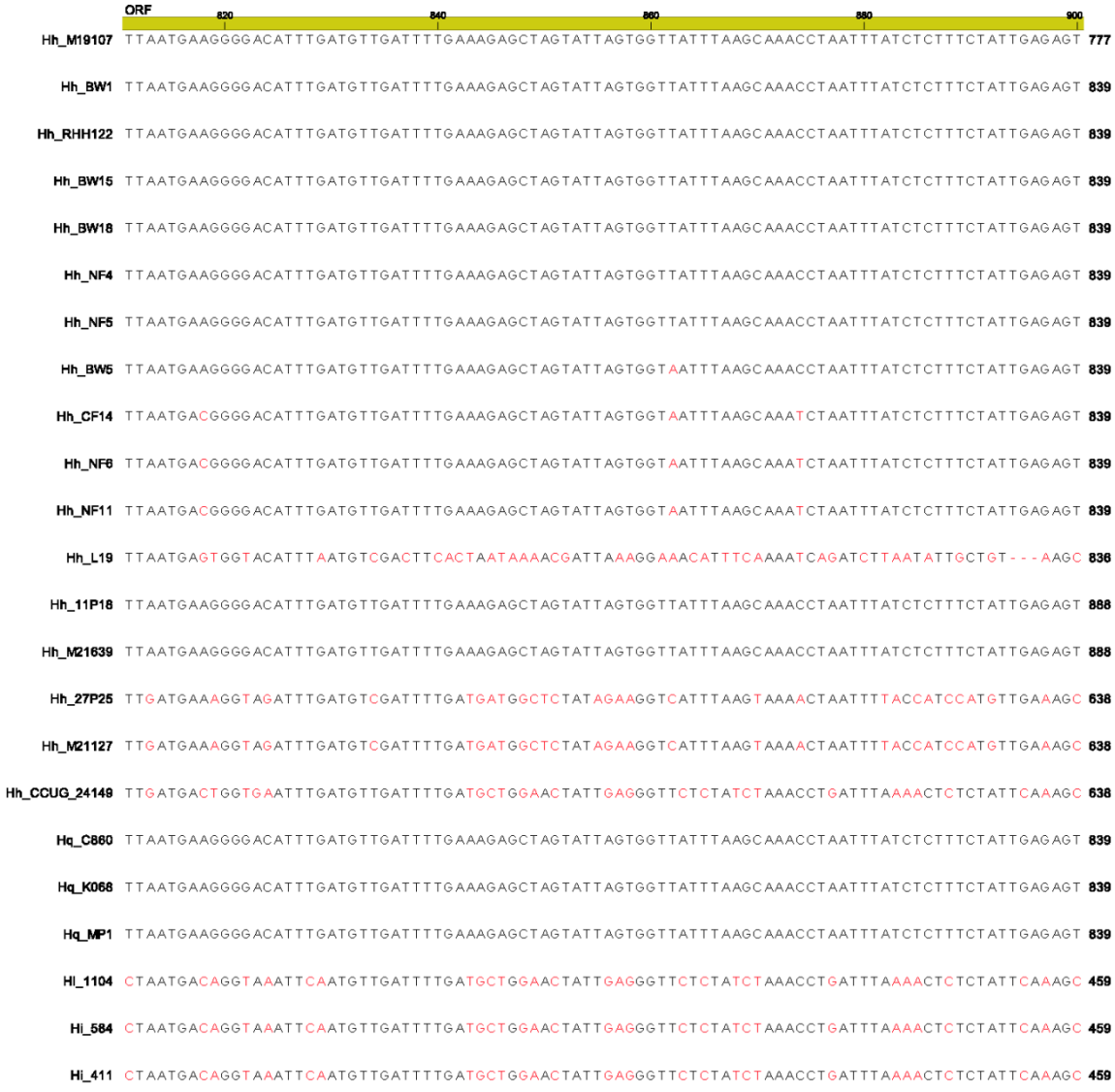
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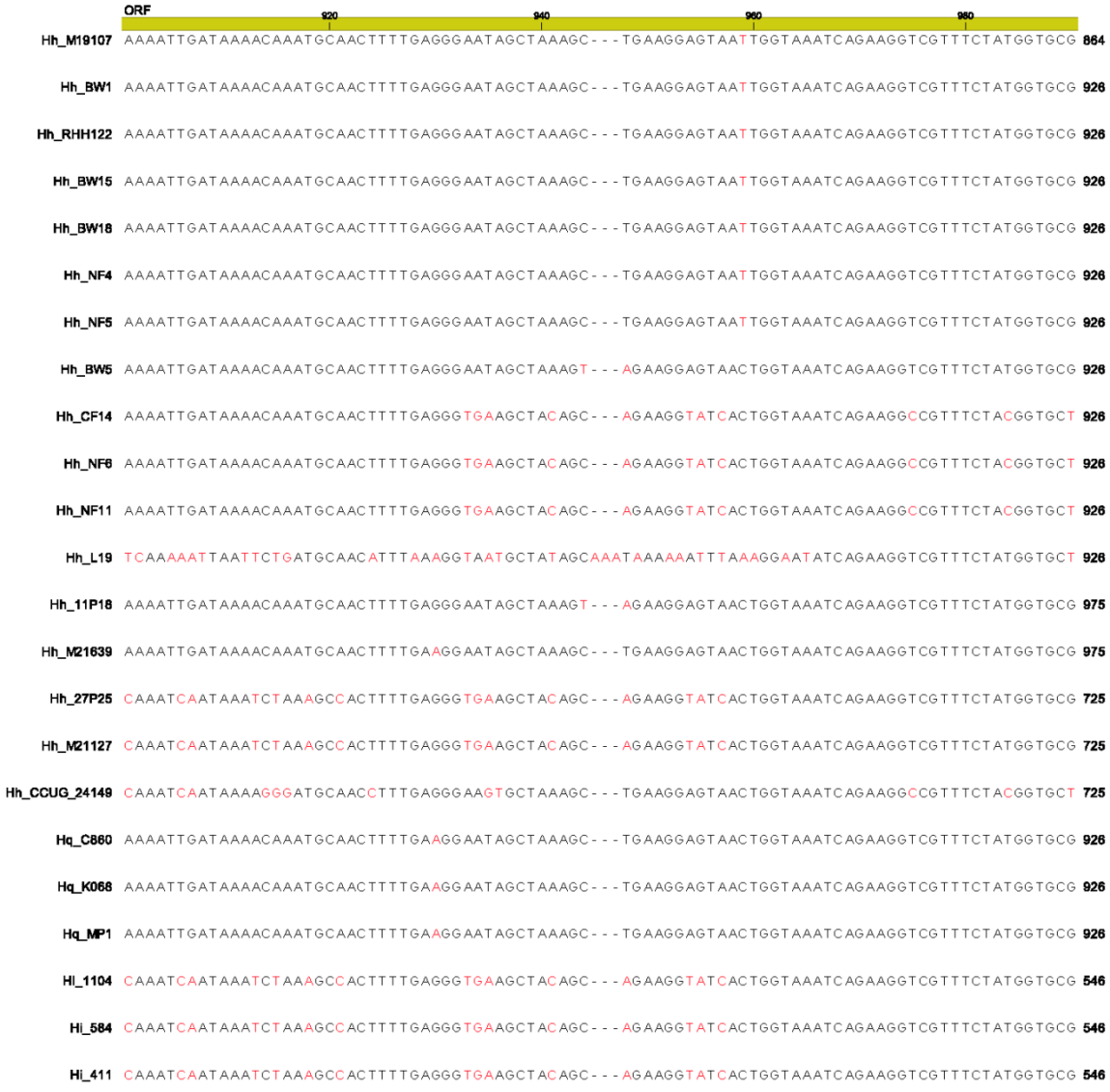
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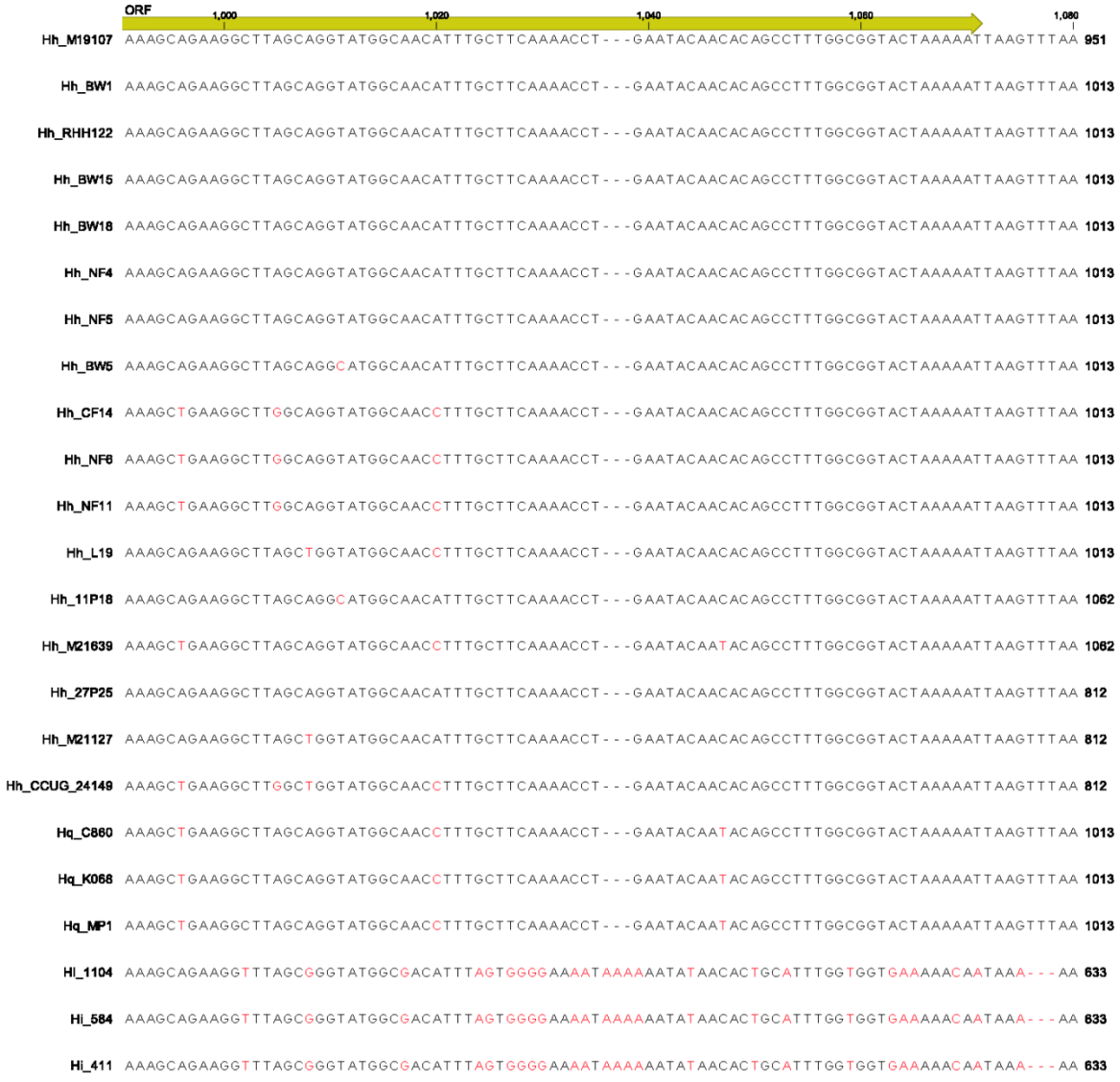
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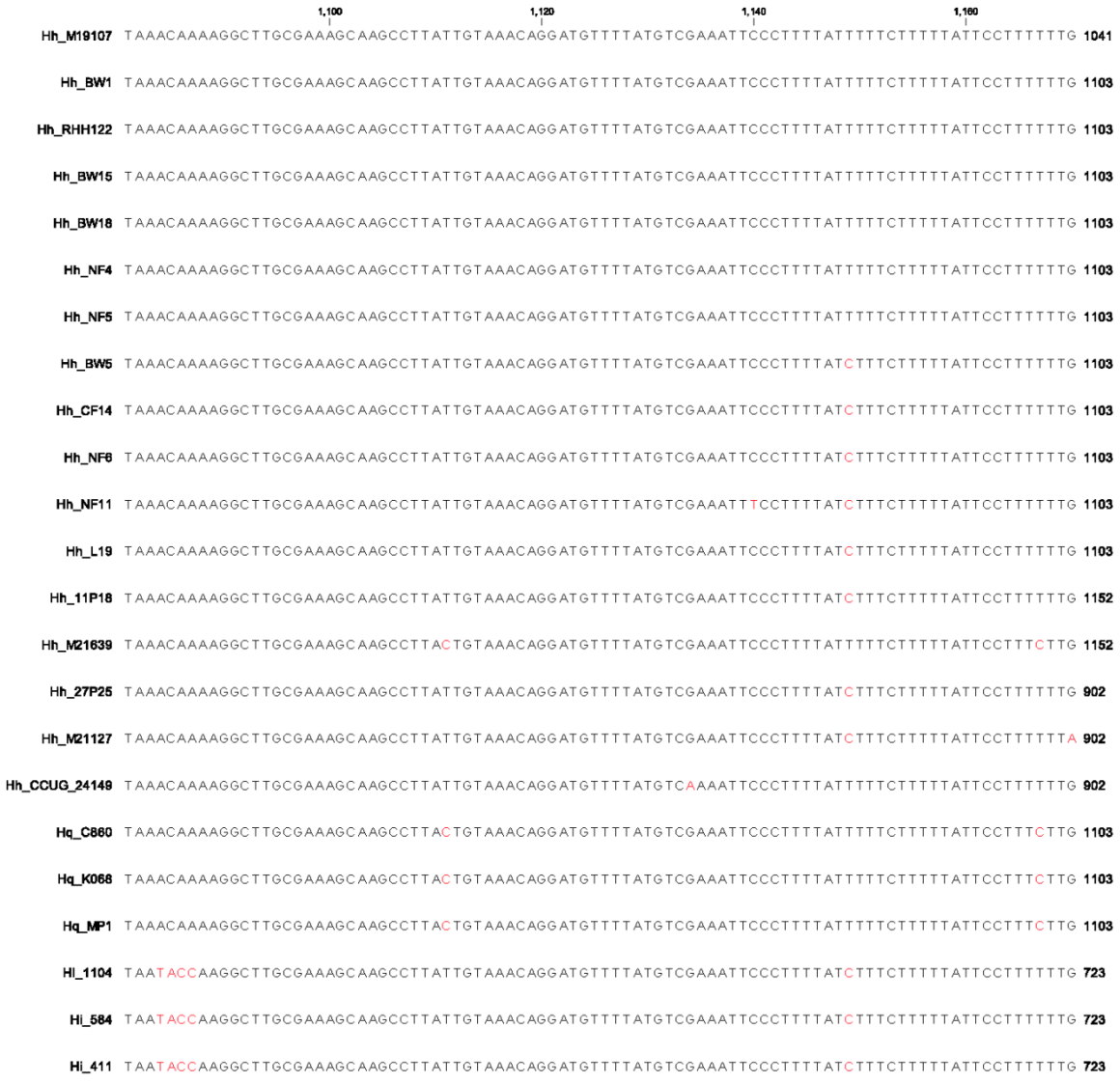
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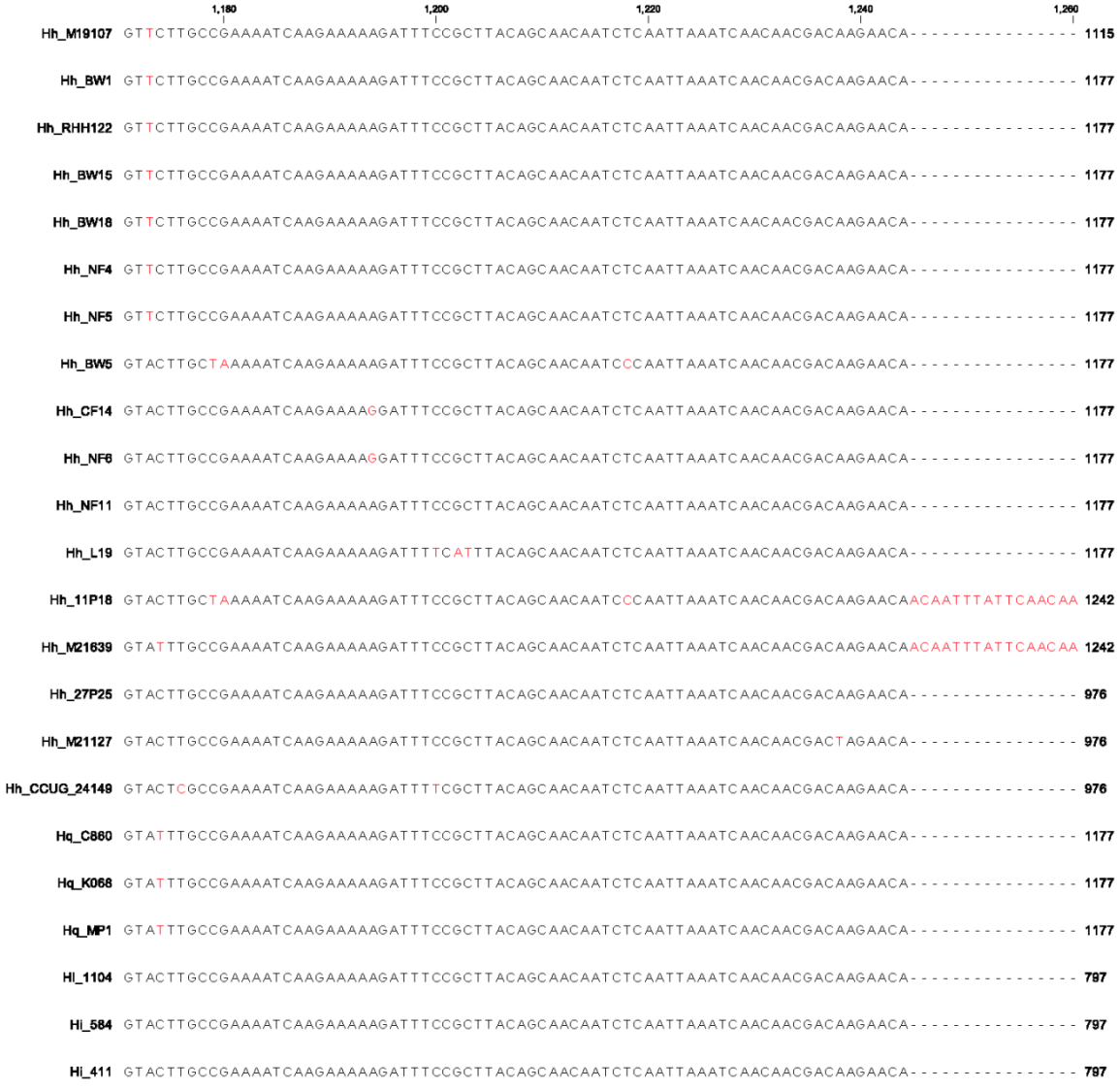
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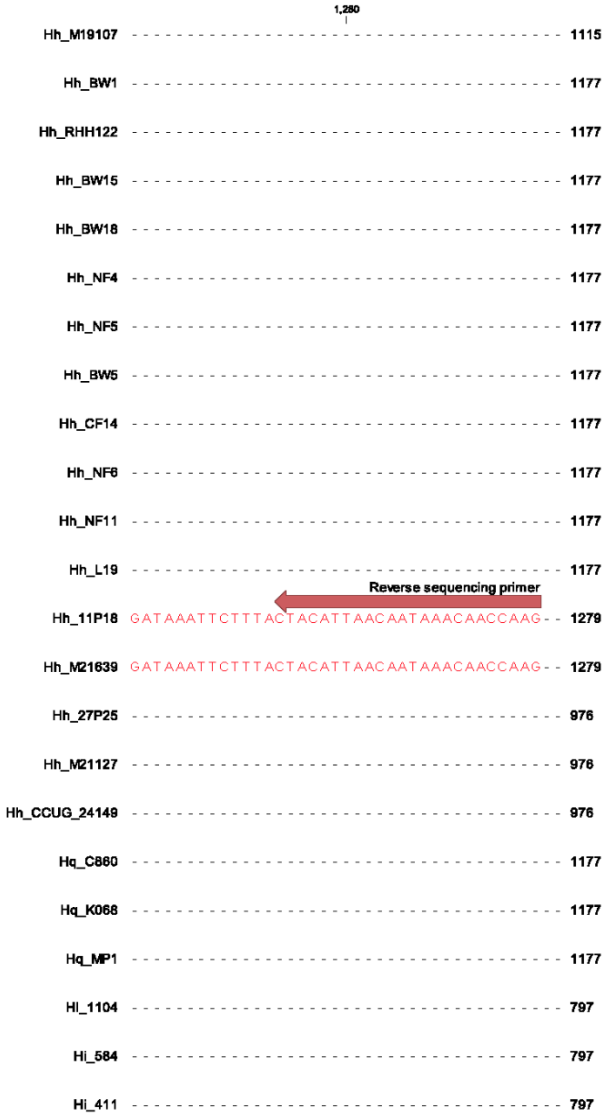
Hi_411 GTACTTGCCGAAAATCAAGAAAAAGATTTCCGCTTACAGCAACAATCTCAATTAAATCAACAACGACAAGAACA---------- 797


1,280
Hh_M19107 1115
Hh_BW1 1177
Hh_RHH122 1177
Hh_BW15 1177
Hh_BW18 1177
Hh_NF4 1177
Hh_NF5 1177
Hh_BW5 1177
Hh_CF14 1177
Hh_NF6 1177
Hh_NF11 1177
Hh_L19 1177
Reverse sequencing primer
Hh_11P18 GATAAATTCTTTACTACATTAACAATAAACAACCAAG 1279
Hh_M21639 GATAAATTCTTTACTACATTAACAATAAACAACCAAG 1279
Hh_27P25 976
Hh_M21127 976
Hh_CCUG_24149 976
Hq_C860 1177
Hq_K068 1177
Hq_MP1 1177
Hl_1104 797
Hi_584 797
Hi_411 797